Characterisation of starch in Musa fruits

Steele, A. F.

Award date:
1997

Awarding institution:
University of Bath

Characterisation of Starch in *Musa* Fruits

Submitted by A. F. Steele

for the degree of PhD

of the University of Bath

1997

University of Bath,

Claverton Down,

Bath BA2 7AY

Natural Resources Institute,

Central Avenue, Chatham Maritime,

Chatham, Kent ME4 4TB

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

To Mum and Dad, Trevor, Joanne and Sards.

Index of Contents

List of Figures

List of Tables

List of Plates

Abbreviations and Symbols

AOAC	Association of Official Analytical Chemists
ADP	Adenosine diphosphate
AM	Amylose
AP	Amylopectin
~	Approximately
ATP	Adenosine triphosphate
C_i	Concentration of carbohydrate
Dist.	Distilled
DP	Degree of Polymerisation
DP_w	Weight-average degree of polymerisation
d.w.b	Dry weight basis
FHIA	Fundacion Hondureña de Investigación Agrícola
GBSS	Granule-Bound Starch Synthase
GPC	Gel Permeation Chromatography
H-bonding	Hydrogen-bonding
HPSEC	High-Performance Size-Exclusion Chromatography
Int.	Intermediate
M_g	Geometric mean particle size
M_w	Weight-average molecular weights
MW	Molecular Weight
n	Number of replicates
OLM	Optical Light Microscope
pers. comm.	Personal communication
PVP	Polyvinylpyrrolidone
PTFE	Polytetrafluoroethylene
RH	Relative Humidity

SBE	Starch Branching Enzyme
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscope
SGBSS	Starch Granule-Bound Starch Synthase
σ_g	Geometric mean standard deviation
SSS	Soluble Starch Synthase
TEM	Transmission Electron Microscope
UDP	Uridine diphosphate
var.	Variety

Units

BU	Brabender Units
BV	Blue Value
SNU	Stirring Number Units
v/v	Volume/volume
w/v	Weight/volume

Acknowledgements

I would like to thank my supervisors Dr. J. Orchard and Dr. K. G. Moore. I am also grateful to Dr. H. Wainwright for his supervision during the first year of this research programme.

I would particularly like to thank the following people for their friendly advice and assistance, and for the use their laboratory equipment and facilities: Dr. A. M. Smith (John Innes Institute, U.K.); Dr. M. Debet, Mr. D. Cooke and Dr. M. J. Gidley (Unilever Research Ltd., U.K.); Mrs. U. Potter (University of Bath); and Dr. A. Fernandez and Dr. M. H. Ong (formerly of the University of Nottingham).

Thank you to Dr. D. Rees and Mr. T. R. Steele for their assistance in the preparation of this thesis. I am also very grateful to the many other people who I have met throughout this research programme who have shared their scientific knowledge and provided me with inspiration and encouragement.

Abstract

Musa fruits are important starchy staples in many developing countries, where they are mostly cooked before consumption. Starch is the main component of unripe *Musa* fruits, and starch determines the cooking qualities of the *Musa* fruits, or flours produced from these fruits. The primary objective of this research programme was to examine the physical, structural and molecular characteristics of the starches of different *Musa* types (dessert bananas, plantains and cooking bananas) in relation to their cooking qualities. A better understanding of these factors which influence the functional characteristics of the starches would ultimately be beneficial to breeders for the development of improved *Musa* germplasm.

In this research programme, flours were produced and starches were isolated from the fruits of different *Musa* types. The physical, chemical and chromatographic laboratory techniques which were used to study the physical and structural characteristics of the *Musa* starches included scanning electron microscopy, transmission electron microscopy, optical light microscopy, powder X-ray diffraction, the Coulter Counter, an iodimetric assay, and gel permeation chromatography. The Brabender Viscoamylograph and the Rapid Viscoanalyser were used to study the functional (cooking) properties of the *Musa* starches.

The starch granules of the different *Musa* types were similar in shape (oyster-shell and irregular) and size (diameters ranged from 16.9-22.1 μm). The structural arrangement of the polymers within the starch granules of plantain and dessert banana resembled that of the C-type starch crystalline pattern. The crystallinities of the starch granules of unripe dessert banana and plantain were similar (between 29.5-31.6%). The molecular characteristics of the amylopectin component in the starches of the different *Musa* types were also similar. Minor differences were observed between the amylose contents of the

different *Musa* starches; cooking banana starch and plantain starch had higher amylose contents (21.6-23.9%) than dessert banana starch (16.8-17.9%).

The experimental results showed differences between the cooking properties of the starches and flours of the different *Musa* types. Unlike that of the cereal starches (which were used for comparison with the *Musa* starches), the amylose contents of the *Musa* starches did not relate consistently to their pasting characteristics. The results suggested that the different pasting characteristics between the different *Musa* types were probably due to molecular differences between the starches, though no molecular differences were found between the different *Musa* types using gel permeation chromatography. Therefore, it is proposed that the fine molecular characteristics of both the amylose and the amylopectin polymers in *Musa* starches requires further investigation, using more sensitive techniques than gel permeation chromatography, to fully explain the reasons why different *Musa* types behave differently during cooking.

The biosynthesis of starch is under specific genetic instruction, and it is possible that species and cultivar differences may lie within the starch biosynthetic enzymes. Preliminary experiments on starch biosynthesis in *Musa* investigated the molecular weight and immunological cross-reactivity of the major starch granule-bound starch synthase (SGBSS). The experimental results indicated that there were similarities in the major SGBSS protein between the different *Musa* types and different plant species. It is possible that differences may lie in other starch synthases, or in the starch branching enzymes, or both, or in the levels of expression of these starch biosynthetic enzymes in different *Musa* types.

Chapter 1

General Introduction

Bananas and plantains are important starchy staples in developing countries, particularly in parts of Africa (Samson, 1986; Purseglove, 1972). The total combined world production of bananas and plantains is over 76 million tonnes, of which exports (mainly of the Cavendish dessert banana) to the richer nations is approximately 11 million tonnes (Price, 1995). The rest of *Musa* production is made up of a wide variety of banana and plantain varieties produced by subsistence farmers or smallholders and their families, either for home consumption or local trade.

The three major naturally occurring edible *Musa* types (*i.e.* the principal clones) comprise plantains, dessert bananas and cooking bananas. There are many different cultivars (*i.e.* cultivated varieties) within each *Musa* type. Plantains are mostly cooked before consumption unless they are very ripe and soft, when they are eaten raw as dessert fruits; dessert bananas are consumed as fresh fruits when ripe, when they are sugary and easily digestible (Samson, 1986; Purseglove, 1972); and cooking bananas are cooked as vegetables when unripe (Rodriguez-Sosa *et al.*, 1977; Rodriguez-Sosa and Parsi-Ros, 1984).

In the last twenty years, the production of bananas and plantains has continued to decline as a result of decreasing soil fertility, yield decline phenomena, pest problems (*e.g.* weevils, nematodes) and disease (such as the wide-spread leaf spot disease known as Black Sigatoka or *Mycosphaerella fijiensis*) (cited by Dadzie, 1995). There are a number of banana and plantain breeding programmes running throughout the world which aim to produce new material (germplasm and new hybrids). Disease resistance and productivity are the main objectives in breeding programmes. However, it is essential that the characteristic banana and plantain physical and end-use properties are preserved in the new disease-resistant hybrids for them to be acceptable by local consumers. To assist in the breeding selection process, there is a need to understand the fruit characteristics which

underlie and determine the cooking properties, and textural and nutritional qualities of bananas and plantains. The major factors which are known to influence these end-user attributes of the fruits are their starch contents and starch characteristics.

Starch is a defined molecular component of plant cells and is made up of a three-dimensional granule composite, with a polymeric structure. This structure is specific to the particular plant species, and can differ between different cultivars of the same species. It would benefit *Musa* breeders to better understand the physical, molecular and structural characteristics of the starches of different *Musa* types for them to be preserved in the new hybrids. It is also important to understand the relationship between these starch granule characteristics and the behaviour of the starch granules during cooking (*i.e.* their functional behaviour) in the different *Musa* types, because bananas and plantains are mostly cooked before consumption. This information could help to explain why the different *Musa* types are utilised for their different properties.

Starch is laid down during its biosynthesis under the instruction of genetic factors and biosynthetic enzymes. Thus, the enzymes of starch biosynthesis may be responsible for contributing to differences in the properties of starches between different plant species and different cultivars. If the physical, molecular and structural differences of starches are found to be related to the enzymes of starch biosynthesis (*e.g.* the relative abundance or rates of activity of these enzymes) in the different *Musa* types and cultivars, these enzymes could be targeted as genetic markers and used to predict starch quality in the fruits of new *Musa* hybrids. The potential would then exist for developing disease-resistant hybrids with the desirable fruit quality characteristics of dessert bananas, cooking bananas and plantains.

Chapter 2

Literature Review and Aims of this Research

2.1 Introduction

As a preliminary to the experimental programme, a literature review was prepared to highlight the key aspects of the information available on *Musa* (section 2.2), the characteristics and biosynthesis of the starch granule (sections 2.3 and 2.4), and the behaviour of starch during cooking (section 2.3), in the context of this research programme.

2.2 *Musa*

2.2.1 Geographical Origin of Bananas and Plantains

The botanical origin of bananas and plantains is in Indochina and South East Asia, where the greatest diversity of the wild *Musa* species is found (Simmonds, 1962). Tens of thousands of years ago, a wide range of *Musa* types arose with the development of agriculture. The earliest historical records of the banana came from India around 600-500 B.C., though the crop was almost certainly present in the country for many millennia before that (Simmonds, 1966). Introduction of bananas to East Africa was probably via Madagascar around A.D. 500. From there, banana cultivation spread across the tropical centre of the continent to the west coast, reaching the Mediterranean in A.D. 650. Polynesian travellers took bananas to the Pacific around A.D. 1000 (Simmonds, 1966). The first of many introductions into the Caribbean and tropical America occurred in 1516, when bananas were brought to Hispaniola (Haiti) from the Canary Islands. This region is where most of the bananas exported to temperate countries are now produced (Purseglove, 1972).

2.2.2 Classification

All edible banana and plantain cultivars belong to the *Musa* genus of the family *Musaceae* (Palmer, 1971). The *Musa* genus contains four sections: *Eumusa*, *Rhodochlamys*, *Australimusa* and *Callimusa*. *Eumusa* is the largest, most diversified, and geographically

most widely distributed section of the *Musa* genus, extending from southern India to Japan and Samoa (Purseglove, 1972). *Callimusa* and *Rhodochlamys* are of ornamental interest only. The *Australimusa* cultivars are utilised across a large area of the Pacific as a cooked vegetable (Palmer, 1971). 'Dessert banana', 'plantain' and 'cooking banana' are names given to groups of closely related *Musa* clones (or types), and each is comprised of many different cultivars. For simplicity, this thesis will refer to dessert bananas, plantains and cooking bananas as different *Musa* 'types'.

2.2.2.1 Genomes

Banana and plantain cultivars are natural hybrid polyploids (diploids, triploids and tetraploids).

	Number of Chromosomes in the *Musa* Cultivar [Haploid Pollen (n) = 11]		
	Diploid Cultivar	**Triploid Cultivar**	**Tetraploid Cultivar**
Chromosome Set (2n)	22	33	44

Nearly all edible banana and plantain cultivars are derived from the two wild diploid species *Musa acuminata* (genome AA) and *Musa balbisiana* (genome BB) (Simmonds, 1966; Palmer, 1971). Cultivated bananas are often described by genome nomenclature, whereby the *Musa* type (or clone) is followed by a group of letters indicating the level of ploidy and the contribution of the two wild diploid species to its genetic make-up. Most edible bananas (and all dessert bananas currently grown for international trade) are triploid and genomically described as AAA, *i.e.* they carry three sets of chromosomes derived from *Musa acuminata*. Plantains are *acuminata*-dominant

triploid hybrids (genome AAB). Plantains resemble bananas in appearance, but their fruits are larger, coarser, thicker-skinned, and richer in starch (Rašper, 1969). Cooking bananas are *balbisiana*-dominant triploid hybrids (genome ABB). Other genomic combinations include AB, AAAA and ABBB, though they occur less frequently. Triploids have been much more successful than diploids and tetraploids because of their greater productivity, vigour, sterility and range of useful genetic variability (Purseglove, 1972).

2.2.2.2 Hybridisation

The development of the domesticated *Musa* fruit occurred when edible forms of *Musa acuminata* migrated outwards from a centre somewhere in Malaysia, accompanied by hybridization and the occurrence of polyploidy (Simmonds, 1966). Hybridization between various sub-species led to a range of diploid cultivars. By a process during meiosis, termed chromosome restitution, diploid cultivars (genome AA) gave rise to triploid types (genome AAA). Experiments have shown that some nearly-sterile edible diploids have a tendency to produce triploid progeny, the products of female restitution, with 22 chromosomes (AA) in the female gamete and 11 chromosomes (A) from the haploid pollen (Purseglove, 1972). Hybridizations in the wild between AAs and *M. balbisiana* (genome BB) gave rise to the various AAB and ABB types now found.

2.2.2.3 Differences Between Genomes

AAA types provide the many sweeter dessert cultivars, whilst AAB and ABB types are starchy cooking varieties. The B genome gives the fruits of plantains and cooking bananas their starchy acidic quality (Simmonds, 1966). In addition, the *balbisiana* genome produces fruits which have a higher dry-matter content, more vitamin C, less liability to phenolic browning, and textures and flavours in the fruits which are not available in pure *M. acuminata*. The ripe fruits of the *M. balbisiana* hybrids are also more suitable for cooking (Purseglove, 1972). The B genome, inherited from crosses from the diploid

M. balbisiana cultivar, is also believed to be provide more drought and disease resistance than the A genome inherited from the diploid *M. acuminata* cultivar. For this reason, *M. acuminata* types are generally found in the humid tropics, while AB, AAB, and ABB hybrids are better adapted to marginal monsoon conditions (Purseglove, 1972).

2.2.2.4 Terminology

In older literature, it is common to find names given by Linnaeus: *Musa sapientum* for banana and *Musa paradisiaca* for plantain. This terminology, and the indiscriminate use of the terms 'banana' and 'plantain', can lead to confusion (Samson, 1986). The term 'banana' is often used to designate ripe fruits eaten raw and 'plantain' for unripe or ripe fruits which are eaten cooked. This is incorrect as not all *Musa* fruits which are eaten cooked are plantains, in the botanical sense. The *Musa* fruits which were used in the experiments described in this thesis are described in section 3.1.1, *i.e. Musa* type (genome and sub-group).

2.2.2.5 Exploitation of Parthenocarpy

Parthenocarpy (the development of fruit without pollination) and an absence of seeds were the most favourable features selected by man throughout the domestication of the banana, though the mechanisms behind these features are still not fully understood (Price, 1995). Most cultivated types are triploid and parthenocarpic, as they bear two genomes from the edible parent and are male sterile. Varieties are cultivated by vegetative propagation of lateral shoots, described as 'suckers', which grow from the underground corm (or 'rhizome') of the 'mother' plant (Simmonds, 1966).

2.2.3 The Banana Fruit

The banana fruit is a berry and contains many ovules. The pulp develops from the outer edge of the loculi (inner face of the skin) and from swelling of the septa and axis. In the cultivated edible bananas, the fruit develops parthenocarpically (without fertilisation) and edible pulp without seeds is produced (Samson, 1986).

A single *Musa* fruit is termed a 'finger', and a cluster of fruit is described as a 'hand' (Purseglove, 1972). The size, shape, skin colour and flavour characteristics of *Musa* fruits vary between different cultivars (Samson, 1986; Purseglove, 1972). Mature *Musa* fruits may vary from 6-35 cm in length and 2.5-5 cm in diameter and they may be green, yellow or red. At harvest, when the mature unripe fruit is cut for export, it weighs half that of the fully ripe fruit (Purseglove, 1972).

2.2.3.1 Ripening of the Banana Fruit

Bananas are climacteric fruits like apples, pears, peaches, tomatoes and avocados (Dominguez-Puigjaner *et al.*, 1992), *i.e.* they characteristically have a growing and maturing period during which they remain firm, green, starchy and astringent. This period is known as the preclimacteric or 'green life'. Rhodes (1970) defined the climacteric as 'a period in the ontogeny of certain fruits, during which a series of biochemical changes is initiated by the auto-catalytic production of ethylene, marking the change from growth to senescence and involving an increase in respiration and leading to ripening'. Ethylene is believed to regulate the expression of the genes involved in ripening (Dominguez-Puigjaner *et al.*, 1992).

During ripening, the respiration rate of the fruit rises to a climax and the fruit softens, loses chlorophyll to reveal yellow pigments, converts starch to sugar, and loses most or all of the astringency. Tannins, which act as inhibitors to the starch degradative enzymes

(cited by Young *et al.*, 1975), are thought to be responsible for the astringency in green banana fruits (Aked and McDowell, 1993). Other protein-like inhibitors of starch degradative enzymes, which disappear during ripening, have also been reported in unripe banana (cited by Halmer and Bewley, 1982).

A characteristic feature of banana fruits is the relatively large proportion of peel (exocarp) tissue, which makes up approximately 80, 40, and 33% of the fresh weight of juvenile, mature and fully ripe fruits, respectively (Palmer, 1971). During ripening, there is a rapid increase in the sugar concentration in the pulp compared to the peel (see below) which contributes to a differential change in osmotic pressure. The peel loses water both by transpiration to the atmosphere and also to the pulp by osmosis, resulting in an increased pulp to peel ratio (Von Loesecke, 1950), thereby contributing to an increase in the fresh weight of the pulp as the fruit ripens. The pulp to peel ratio is often used as an index of fruit maturity (see Appendix Ib). The dry matter content of the fruit also increases during ripening, which has been attributed to transpiration during storage (Von Loesecke, 1950).

According to Desai and Deshpande (1975), the quality and nutritive value of fresh fruits are influenced by physical and biochemical changes that occur during their maturation and storage. The sugar to acid balance is important in giving the fruits their pleasant taste. Generally, when *Musa* fruits are harvested at matured green stage, the pulp pH is high but drops during ripening. Plantains generally have less water and are more acid than dessert bananas (Purseglove, 1972; Forsyth, 1980).

In climacteric fruit such as the banana, carbon is stored largely in the form of starch and the starch content remains high into the ripening period. During ripening, starch is thought to be hydrolysed within the amyloplast (see section 2.3.1) to hexose monophosphates, which are directly transported across the amyloplast envelope (which

maintains its integrity throughout the ripening process) into the cytosol where they are dephosphorylated (Smith and Denyer, 1992). The loss of starch in bananas commences in the locules next to the degenerate ovules and spreads through the locule as ripening progresses (Wainwright and Burdon, 1991; Blankenship *et al.*, 1993). As ripening proceeds, starch is converted almost entirely to CO_2 and sugars (sucrose, glucose and fructose) by starch degradative enzymes (amylases and phosphorylases) (Von Loesecke, 1950; Palmer, 1971). The sugars are responsible for the sweetening of the fruit (as it ripens). During ripening, the amounts of proteins and sugars in the plantain fruit increase by ~½% and 16% (fresh weight), respectively (Ketiku, 1973). The decreasing starch content during ripening is more pronounced in the pulp than the peel (Ketiku, 1973). The starch content of green *Musa* fruits is about 20% (fresh weight) and this drops to about 1-2% in the ripe fruits (Simmonds, 1966). The starch is converted less rapidly to sugars in starchy types, such as plantains; the starch content in ripe plantains is about 6% higher than in ripe dessert bananas (Simmonds, 1966). During ripening there is a close correlation between the starch-to-sugar ratio and peel colour (cited by Forsyth, 1980). The subjective evaluation of the peel colour of banana fruits, by comparing banana fruit with colour plates, is another widely used fruit maturity index measurement (see section 3.2.1). Purseglove (1972) reported the pulp of the ripe banana (approximately two thirds of the whole fruit) to contain:

water	~70%
carbohydrate	27.1%
protein	1.2%
fat	0.3%
fibre	0.5%
ash	0.9%

In *Musa* fruits, the firmness of the pulp decreases as ripening continues. This softening or loss in pulp firmness during ripening has been associated with the breakdown of starch to form sugar, the breakdown of the cell walls, or reduction in the middle lamella cohesion due to the solubilisation of pectin (Palmer, 1971), and the movement of water from the peel to the pulp during ripening due to osmosis.

2.2.4 *Musa* Flour and Its Economic Importance

Processing is a recognised way of preserving fruit, though most *Musa* fruits are eaten raw when ripe, or as a cooked vegetable, and only a very small proportion are processed to obtain a storable product. In general, processed *Musa* products do not contribute significantly to the diets of the millions of people who eat bananas, cooking bananas and plantains. However, in some areas, the processed or preserved products are important when food is scarce (Dadzie, 1995) (see below).

Besides powder, canned slices and chips, flour is one of the more common and widespread processed products into which *Musa* fruit can be made. *Musa* flour is produced from dried pulp which has been ground or blended into a powdery material. It can be made from green unripe dessert banana, cooking banana or plantain. The dried pulp slices may be stored and only converted into flour when needed, since the flour tends to lose its flavour rapidly and may absorb moisture (Dadzie, 1995). Kayisu *et al.* (1981) reported the starch content of unripe (green) dessert banana flour and ripe dessert banana flour ('Valery' var.) to be 78.0 ± 0.4% and 16.1 ± 0.5%, respectively. Thus, flour made from unripe *Musa* fruits is mainly composed of starch, and when an aqueous *Musa* flour suspension is cooked, the cooking behaviour can be considered to be essentially due to the starch granules in the flour suspension.

Baked goods are traditionally prepared using cereal flours such as wheat or rye. However, to reduce the need to import wheat from overseas, third world countries are being encouraged to produce flour from staples grown in tropical countries as partial or complete substitutes for cereal flours in baked goods (Bamidele *et al.*, 1990). These substitutions can be both economical and nutritionally beneficial (Rodriguez-Sosa *et al.*, 1977; Suntharalingam and Ravindran, 1993). Rejected *Musa* fruits which are unsuitable for export can be used as flour substitutes. For many years, research has been carried out on the production of consumer acceptable *Musa* flours. Flours with a high degree of product acceptability and stability during storage have been produced from ripe and unripe fruits using cabinet and oven drying techniques (Rodriguez-Sosa *et al.*, 1977; Ukhun and Ukpebor, 1991).

2.2.5 Banana and Plantain Breeding: The Importance of Fruit Quality

There are many diseases which threaten commercially important *Musa* cultivars. The chemical control of banana diseases is both expensive and hazardous to health. However, *Musa* breeding and the genetic manipulation of tissue *in vitro* (see Vuylsteke, 1989) offer an alternative approach. The principal objective for *Musa* breeding programmes is disease resistance, though the new varieties must also retain all of the essential characteristics of the current varieties, such as plant vigour, suitable morphology, productivity and fruit quality.

The biotechnology of hybrid production is technically difficult, and breeders have so far been largely unsuccessful in combining disease resistance, along with improved or equivalent agronomic and fruit quality characteristics, into one variety (Ortiz *et al.*, 1995). Usually, the new resistant hybrids are not as acceptable to consumers as the original varieties. Clearly, more emphasis needs to be placed on breeding for quality.

2.3 Starch

Despite the importance of *Musa* cultivars as tropical food crops, comparatively little is known about the properties of their starches because these crops have been used almost entirely for the preparation of food at the domestic level (Rašper, 1969). Therefore, the following sections discuss starch and starch biosynthesis in plants generally, with some reference to reports on *Musa* starch in the literature.

2.3.1 Occurrence and Storage of Starch

Starch is the principal reserve carbohydrate of higher plants and occurs as dense water-insoluble granules which are semi-crystalline (Preiss and Levi, 1980). Reserve starch in storage cells is synthesised in amyloplasts. Amyloplasts are extremely fragile double membrane-bound organelles, which are ontogenically related to chloroplasts: amyloplasts develop from proplastids and may develop into chloroplasts (or *vice versa*) (Shannon and Garwood, 1984). The amyloplast is usually about 4-6 μm in diameter at the time of starch-grain initiation (Sterling, 1968). Amyloplasts can enclose one starch granule (*e.g.* pea, maize, wheat, potato, barley), or several granules (*e.g.* rice, oats, cassava, sweet potato). The growing starch granules in the amyloplast are surrounded by a thin layer of stroma which contains the enzymes of starch biosynthesis (Shannon and Garwood, 1984) (see section 2.4).

2.3.2 Physical Characteristics of Starch Granules

The shapes and sizes of starch granules in plants is characteristic for each plant species. The size of starch granules is dependent on the plant tissue, as different tissues contain different sized starch granules. Granular shapes and sizes are also dependent on the maturity or stage of development of the plant at the time of isolation of the starch granules (Preiss, 1992); starch granule sizes are non-uniform in most species, and the average granular size increases with age during starch biosynthesis (Shannon and Garwood, 1984).

Environmental conditions, such as seasonal and climatic variations, can also affect the average starch granule diameter (Asaoka *et al.*, 1991). The diameter of various kinds of starch granules can range from 1-2 μm to about 200 μm, and they may be spherical, egg-like, or irregular in shape (MacMasters, 1964). Lii *et al.* (1982) reported banana starch granules to range from oval to irregular in shape, and from 20-60 μm in size.

Starch granule sizes and their numbers can be measured with a Coulter Counter. This instrument records the magnitude of the electrical resistance created when (starch) particles in electrolyte solution are forced to flow through a small aperture between two electrodes. The magnitude of the resistance is essentially proportional to the volume of the particles.

The physical characteristics (shape and size) of *Musa* starch granules are examined in Chapter 4.

2.3.3 Components of Starch Granules

The major components of starch granules are amylose and amylopectin (see section 2.3.3.1). Starch granules also contain small amounts of non-carbohydrate components, particularly lipids, protein, and phosphates which may affect the behaviour of the starch granules in various applications. The *Musa*, tuber (potato) and root (tapioca) starches contain only a very small percentage of fatty substances (≤0.2%, w/w), whereas the common cereal starches (corn, wheat, rice, and sorghum) contain 0.6-0.8% (w/w) fatty substances (Kayisu *et al.*, 1981; Swinkels, 1985). Lipids in (cereal) starch granules exist as amylose-lipid inclusion complexes, which repress swelling and solubilisation of the starch granules (Swinkels, 1985) when cooked in an aqueous solution. Starch granules usually contain 10-20% (w/w) moisture, under normal atmospheric conditions, which depends on the relative humidity (RH) of the atmosphere in which they have been stored

(Swinkels, 1985). The following table shows the chemical composition of green banana starch, as reported by Kayisu *et al.* (1981):

moisture	10.8 ± 0.2%
protein	0.2 ± 0.0%
ash	0.02 ± 0.0%
lipid	0.2 ± 0.0%

(± represents Standard Deviation, $n \geq 2$)

2.3.3.1 Major Polymer Components of Starch Granules

Starch is primarily composed of two high molecular weight biopolymers: amylose (AM) and amylopectin (AP). The starch granule generally contains 25-35% AM and 65-75% AP (Preiss, 1992), though the generalised granule composition is subject to great interspecific and developmental variation (Smith and Martin, 1992). Environmental factors also have an effect on the composition of the starch granule, though these effects are not as large as those associated with cultivars or cultivar maturity (Shannon and Garwood, 1984). Most of the functional properties of starch granules are due to the relative proportions of AM and AP, their molecular structure, and the organisation of these starch polymers within the granule.

Eggleston *et al.* (1992) reported low AM contents for their unripe plantain and unripe cooking banana starches (9.11-17.16%). However, Berríos and González (1971) found that the AM contents of green banana (*Musa sapientum*) and green plantain (*Musa paradisiaca*) were 25% and 27%, respectively. The AM contents of green unripe dessert banana starches have been reported as 16% (Kayisu and Hood, 1981) and 20% (Garcia and Lajolo, 1988).

In some plant varieties, a minor third component of starch (or fraction), referred to as intermediate fraction or anomalous AP may also be present in the starch granules (Preiss, 1992). The type and amount of intermediate material in different starches varies considerably, but, for the mature starch, appears to depend upon the AM content of the starch (particularly for the high-AM starch varieties of pea, maize and barley) (Banks and Greenwood, 1975). The structure of this intermediate fraction is not yet fully understood, though various starch researchers have reported the presence of this intermediate fraction with a structure intermediate between those of AM and AP (*e.g.* Hizukuri *et al.*, 1989; Fuwa *et al.*, 1992; Banks *et al.*, 1974; Inouchi *et al.*, 1987). Further research is required to determine the precise origin(s) of the intermediate fraction. Banks and Greenwood (1975) suggested that the intermediate fraction may supply the answer to that most puzzling of questions: namely the manner in which the components of starch are synthesised. There is evidence of an intermediate fraction in the *Musa* starches in Chapter 5.

2.3.3.2 Formation of Amylose and Amylopectin

Starch structure is genetically controlled, though the regulation of these genes may be complex. The means by which plant genetic information is translated into specific granule morphology and the mechanism by which starch granules are initiated in storage organs are unknown. One proposal for the formation of AM and AP is an accumulation of starch molecules in specific regions of the stroma in an unorganised 'droplet' form, which then spontaneously crystallise to form a nucleus around which the granule develops (cited by Smith and Martin, 1992).

Each plant tissue during growth produces AM and AP polymers simultaneously and in a highly specific proportion (Preiss and Levi, 1980). In a starch granule, the hilum is the original growing point of the granule. It can be seen at the centre of the 'Maltese Cross'

(see section 2.3.4) under the polarised optical light microscope. Depending on the species of starch (Schoch and Maywald, 1956), the hilum is a central or eccentric point in the granule around which layers of the AM and AP polymers are deposited. If the layering of the starch polymers is uniform, the hilum is in the centre of the grain (*e.g.* maize starch), but if it is uneven, the hilum may be to one side (*e.g.* potato starch). Lii *et al.* (1982) found that banana starch granules exhibited eccentric hila (see Chapter 4). The hilum is probably less organised than the rest of the granule, and contains non-polysaccharide material from the amyloplast (French, 1984).

2.3.3.3 Amylose

AM is essentially a linear chain (with some branching) of α-(1→4)-linked α-D-glucopyranosyl residues, approximately 1000-10,000 units long (Preiss and Levi, 1980; French, 1984) (see Figure 2.3.1). AM has a typical molecular weight of approximately 10^5 to 10^6. The degree of branching of AM is much less than that of AP (see section 2.3.3.4). The essentially linear nature of AM confers upon it the ability to form complexes with fatty acids, low molecular weight alcohols and iodine (Preiss, 1992). AM adopts a helical inclusion complex with iodine to give a characteristic blue colour (wavelength of maximum absorbance, λ_{max}, 620-680 nm), which is a diagnostic tool for measuring the proportion of AM in any starch (Swinkels, 1985) (see Chapter 5).

AM in solution behaves as a random coil and, while there may be some helical character present, its structure is very loose. The helical nature results not only from intra-molecular H-bonding, but is also due to the geometry of the α-(1→4)-D-glucano-pyranosidic linkages. When an aqueous starch suspension is cooked and then cooled, depending on the temperature, concentration and the degree of polymerisation, AM can spontaneously crystallise from aqueous solution to form a 3-D gel network. This process

leads to increased viscosity and is known as retrogradation (Preiss and Levi, 1980) (see section 2.3.6).

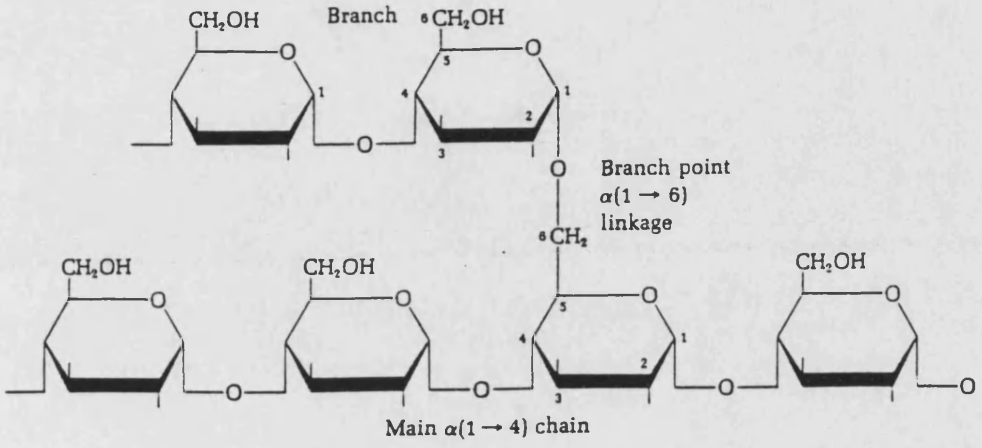
2.3.3.4 Amylopectin

AP is a highly branched polymer of α-D-glucopyranosyl units (see Figure 2.3.1). About 4-5% of the glucosidic linkages are α-1→4, and between branches there are ~20-25 units (Banks and Greenwood, 1975; Manners, 1989). The constituent chain profile of AP may be deduced by enzymic debranching, followed by a molecular size sorting technique such as Gel Permeation Chromatography (GPC) (see Chapter 5).

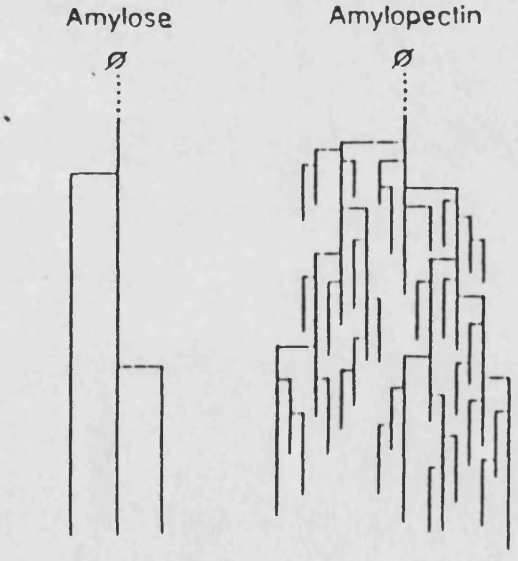
Unlike AM, AP does not form complexes and AP forms a reddish-purple colour with iodine in solution (λ_{max} 550 nm). AP is stable in aqueous solution and whereas AM readily forms H-bonds in solution to form rigid gels, AP has limited H-bonding due to its branching and remains more fluid (Manners, 1968). When an aqueous suspension of AP is heated and then cooled, retrogradation is slower than that of an aqueous suspension of AM (Van Soest *et al.*, 1994).

A typical AP molecule is approximately 2000-4000 Å long and 100-150 Å in diameter, and has a typical molecular weight of 10^7 to 10^8 (Banks and Greenwood, 1975; French, 1984). The molecular structure of AP can be described in terms of various parameters, *e.g.* average chain length, exterior and interior chain lengths, ratio of A- to B-chains (see below), and chain profile (Manners, 1989).

Figure 2.3.1 I, Structure of α-1→4- and α-1→6-Glucan Linkages in Starch (Lehninger, 1975)



II, Diagrammatic Representation of Possible Structures of Amylose and Amylopectin (Shewmaker and Stalker, 1992)



Ø represents Reducing End

In the proposed model of AP (Figure 2.3.2. I), there are three categories for the component AP chains:-

(i) There is only one C-chain per AP molecule, and it carries the sole reducing group in the macromolecule;

(ii) B-chains are linked to the C-chain and other B-chains by their reducing end groups. B-chains can be linked to one or more A-chains;

(iii) A-chains are linked to the B-chains by their reducing end groups through α-(1→6)-linkages.

The short A-chains are organised as double helices and form clusters at regular intervals throughout the molecule linked together by the longer B-chains. The 'cluster' or 'racemose' model, proposed by Robin *et al.* (1974), is the most widely accepted molecular model for AP. It explains the high viscosity of AP, and proposes that the branches are not randomly distributed along the axis of the molecule but occur in discrete clusters at intervals of ~7-10 μm, separated by relatively unbranched regions (Figure 2.3.2) (cited by Smith and Martin, 1992). The branching pattern in AP is extremely heterogeneous, and the ramified nature of this polysaccharide results in it having a very broad distribution of molecular weights (Banks and Greenwood, 1975). Various workers have reported differences in the AP structure even within the same source which could partially explain the diverse functional properties of these starches (cited by Paredes-López *et al.*, 1994).

In the concept of A-, B- and C-chains, the degree of branching may be expressed as the ratio of A-chains to B-chains (Manners, 1989). In most samples of AP there are more A-chains than B-chains, the ratios ranging from about 1.0: 1 to 1.5: 1 (Manners, 1989) (see Chapter 5).

Figure 2.3.2 I, Structure, Dimensions and Location in the Granule of Part of a Generalised Amylopectin Molecule (Smith and Martin, 1992)

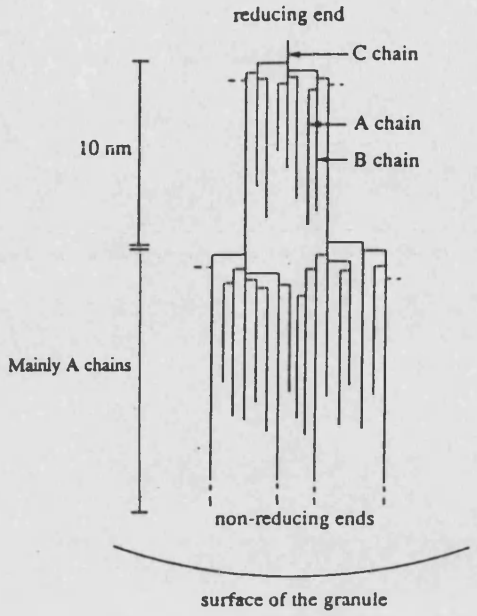
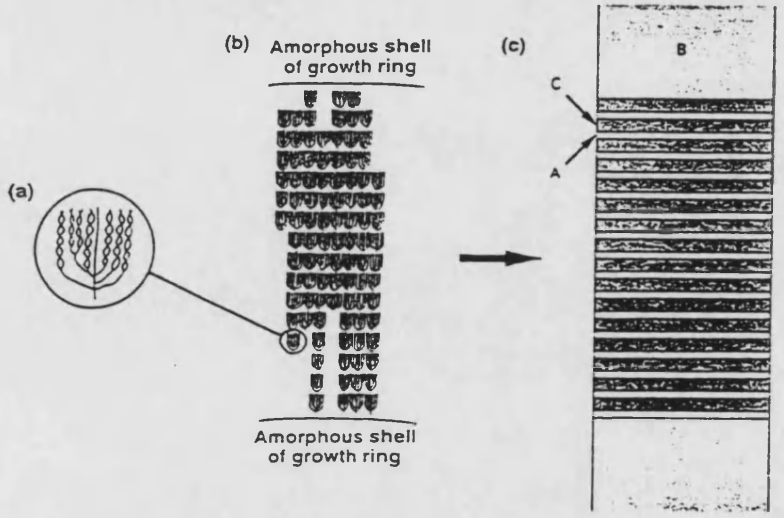


Figure 2.3.2 II, (a) A single AP cluster with double helix formation. (b) Schematic representation of the arrangement of AP molecules within a semi-crystalline growth ring. (c) A model of the structure in terms of a stack of lamellae alternating in crystalline regions C and amorphous regions A, embedded in an amorphous region (Jenkins *et al.*, 1993)



2.3.4 Molecular Organisation of the Starch Granule

AM and AP molecules are radially oriented with their single reducing end groups towards the centre (or hilum) of the granule (Preiss and Levi, 1980; Blanshard, 1987). Growth of the AM and AP molecular chains occurs at the outer, non-reducing chain ends (the non-reducing ends point outwards towards the surface of the granule) (French, 1984; Blanshard, 1987). Under the polarised optical light microscope (OLM), granules exhibit birefringence and many starch granules show internal 'growth rings' or striations, which are concentrically arranged layers of alternating amorphous and crystalline regions (French, 1984) (see Figure 2.3.2. II). The denser layers probably contain crystalline lamellae of AP lying tangentially to the radius of the granule. The crystalline lamellae are formed by the ordered (double-helical) packing of the branching clusters of many parallel AP molecules (Kainuma, 1988). Like the size and shape of starch granules, striations are also characteristic of the variety of starch. The 'Maltese Cross' effect can also be seen using a polarised OLM and is a result of the radial arrangement of the molecules. The arms of the polarisation cross are perpendicular to the growth rings which shows that the optic axes of the starch crystallites, and hence the molecular axes of the starch molecules are aligned perpendicular to the growth ring (Kainuma, 1988).

In cereal starch granules, AM molecules can be present in three forms: as free (constituting an amorphous region), bound radially with lipid, or co-crystallising with AP crystallites in the granule (Blanshard *et al.*, 1984; Blanshard, 1986). Significant components of the AM fraction are located primarily in the amorphous, rather than in the crystalline, phase of the starch granule (French, 1984), though the exact location of this phase relative to the crystalline regions of the granule is not known: there is no sharp demarcation between the crystalline and amorphous phases of starch granules. It is generally thought that some or all of the starch molecular chains run continuously from

one phase to another as the individual AP molecules are larger than the crystallites (French, 1984).

2.3.5 Crystalline Structure of the Starch Granule

There is strong evidence to suggest that it is the AP component which is responsible for the crystallinity of starch granules (French, 1984; Banks and Greenwood, 1975; Chinachoti and Steinberg, 1986), as some varieties of starch which do not contain any AM, such as waxy maize, are crystalline. The short chains of AP are associated in the crystalline regions, while the branch points are located in the amorphous regions of the starch granule. Crystallinity is due to regular packing of adjacent branches within the AP molecule in the form of double helices, giving rise to long range order (or crystallinity) within the granule (Kainuma, 1988; French, 1984; Stevens and Elton, 1971). The semi-crystalline structure of starch will diffract X-rays. Thus, double helices that are packed into regular arrays are shown as broad diffraction peaks superimposed on an amorphous halo on the X-ray diffraction pattern.

In X-ray diffraction, the scattered intensity patterns which are produced can be successfully grouped according to a physical property of the starches. Starch gives a number of distinct types of X-ray diffraction patterns (A-, B- and C-types) (see Figure 2.3.3), which is dependent on the origin and growth conditions of the starch, and also on its experimental treatment (Hizukuri *et al.*, 1961; Nikuni, 1978). The A- and B-patterns represent true crystalline modifications of starch. Hizukuri *et al.* (1961) provided evidence that the C-type structure is a mixture of A- and B- unit cells and therefore a transition or intermediate type between the A- and B-types. Experiments have shown that the average chain length of the AP polymer is the principal determinant of the crystalline polymorphism of granular starches (Hizukuri *et al.*, 1983). Starches which give an A-type diffraction pattern have APs with shorter B-chains than those that give the B-type pattern

(Hizukuri, 1985). In general, in native granular form, the A-type is associated with cereal starches, the B-type with potato and amylomaize starch (and retrograded starch), and certain root and seed starches give C-type patterns (see Figure 2.3.3) (Banks and Greenwood, 1975; Zobel, 1988). In the literature, banana starch has been described as having B-type (French, 1984; Lii *et al.*, 1982) and C-type X-ray diffraction patterns (Sterling, 1968).

According to Sarko and Wu (1978), the unit cells of A- and B-type starches are nearly identical in molecular conformation, though they differ in the crystalline packing of their helices and water contents (see Figure 2.3.4). The 3-D model structures, proposed by Imberty *et al.* (1988) and Imberty and Perez (1988) for A- and B-starches, have a hexagonal unit cell containing 12 glucose residues located in two left-handed, parallel-stranded double helices packed in a parallel fashion, with 4 water molecules located between these helices for A-type starch (Figure 2.3.4. I) (Imberty *et al.*, 1988), and 36 water molecules located between the helices for B-type starch (Figure 2.3.4. II) (Imberty and Perez, 1988).

Pure AM will form A- and B-type crystalline structures with the same inter-planar spacings (*d*-spacings) of the unit cells as observed in starch granules, even those which are mainly composed of AP (Sarko and Wu, 1978). Thus, the AP molecules responsible for the crystallinity of the starch granule are believed to have the same crystalline structure as artificial fibres of A- and B-AM.

The crystallinities and crystalline structures of different *Musa* starches are investigated in Chapter 5.

Figure 2.3.3 X-Ray Diffractometer Tracings from Different Starches (Zobel, 1988)

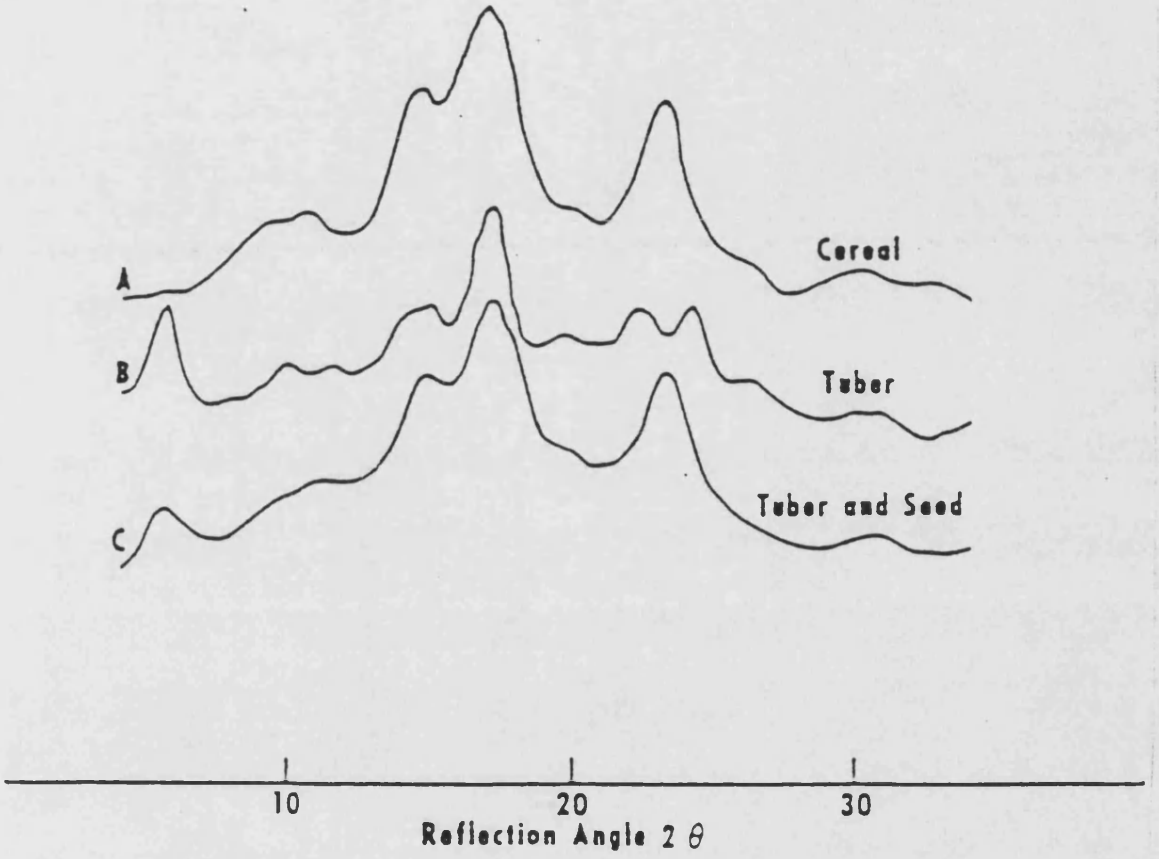


Figure 2.3.4 I, Projection of the Structure of A-Amylose onto the (a, b) Plane. Hydrogen bonds are indicated as dashed lines. The positions of the water molecules are marked by black dots (Imberty *et al.*, 1988).

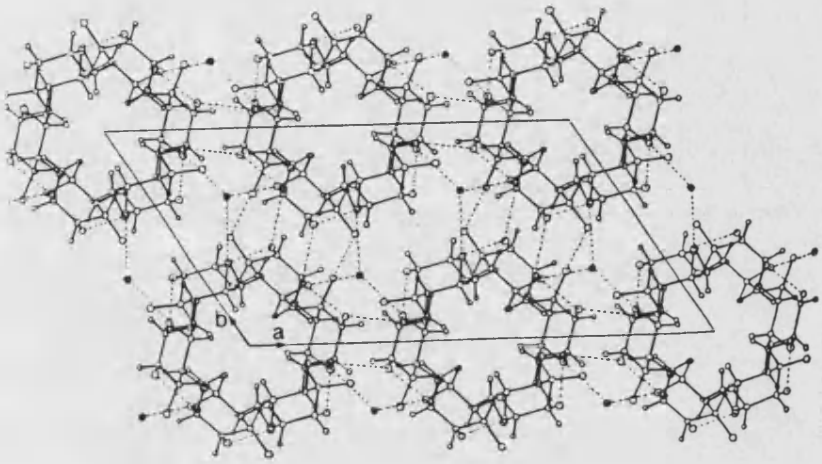
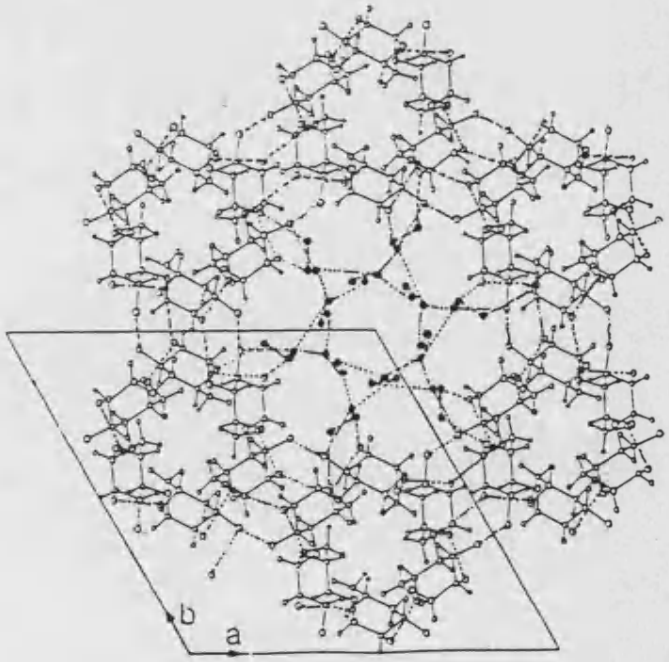


Figure 2.3.4 II, Projection of the Structure of B-Amylose onto the (a, b) Plane. Hydrogen bonds are indicated as dashed lines. The positions of the water molecules are marked by black dots (Imberty and Perez, 1988).



2.3.6 Cooking (or Pasting) Behaviour of Starch Granules

Starch is known to be one of the important factors which determines the cooking quality of various foods (Asaoka *et al.*, 1991). It has also been reported that starch plays an important role in the cooking or pasting behaviour of *Musa* fruits (Rodriguez-Sosa and Parsi-Ros, 1984; Eggleston *et al.*, 1992). The following describes the changes which occur in the conformation and molecular components of starch granules when an aqueous starch suspension is cooked and then cooled.

The pasting process takes place when aqueous starch suspensions are heated above their gelatinisation temperatures. During heating, the viscosity (which refers to the consistency) of the starch suspension increases due to the swelling of the starch granules, consequent on gelatinisation. The size of the increase in viscosity is related to the degree of swelling of the starch granules. As the starch granules continue to swell with increasing temperature, molecular mobility within the granules and further hydration allows molecular reorganisation. The smaller molecules of AM diffuse out of the swollen starch granules (Hari *et al.*, 1989). During continued heating, the swollen starch granules subsequently breakdown, which decreases the viscosity of the suspension.

When the cooked aqueous starch suspension is cooled, the gelatinised starch granule dispersions acquire the consistency of gels. This occurs due to the formation of hydrogen bonds between AM chains and helix-helix aggregation, and eventually the (slow) crystallisation of short AP chains. This phenomenon is known as retrogradation. Retrogradation is an important aspect of starch chemistry in the food industry. X-ray diffraction of retrograded starch shows that crystalline organisation is formed. There is a relationship between the chain length of the AM molecules and the ease and nature of retrogradation (Swinkels, 1985). Retrograded starch, as well as retrograded AM, exhibits the B-type X-ray diffraction pattern. There is an optimal length of AM for retrogradation.

AM molecules longer than this optimum do not readily move into tight association with other chains and have difficulty lining up with their neighbours over long intervals. AM molecules smaller then the optimum length do not associate as completely and are too short to give a gel. A maximum rate of retrogradation (minimum in solubility) occurs when AM has a degree of polymerisation of about 100-200 glucose units (Swinkels, 1985). Retrogradation also depends on many other factors, such as starch type, starch concentration, cooking procedure, temperature, storing time, pH, cooling procedure, and the presence of other compounds (Swinkels, 1985).

The pasting behaviour of starch or flour suspensions can be measured with a Brabender Viscoamylograph. This instrument records the torque required to balance the viscosity that develops when a starch slurry is subjected to a programmed heating and cooling cycle (Zobel, 1984). A similar instrument which records the pasting characteristics of starch-water or flour-water suspensions is the Rapid Viscoanalyser, which uses much smaller volumes of sample than the Brabender Viscoamylograph. Brabender viscoamylogram and Rapid viscogram viscosity profiles show the extent of swelling of starch granules, and the resistances of the swollen granules to dissolution by heat, or fragmentation by shear (Schoch and Maywald, 1968). The viscosity profiles are characteristic and different for each type of starch (Swinkels, 1985).

The pasting characteristics of different *Musa* starches and flour samples are examined in Chapter 6.

2.3.7 Potential for the Genetic Manipulation of the Properties of Starch Granules

Starch has many applications in the food and other industries. The suitability of a starch for a specific use is dependent on its physical properties. As described above, the physical properties of starch granules are due to a number of factors which include the size and shape of the granules, and also the structural properties of the starch granules, such as the ratio of AM and AP, the sizes of these constituent polymers, their degree of branching, and their structural arrangement (Banks and Greenwood, 1975). Genetic manipulation of starch can be used to select or improve desirable quality or compositional characteristics for a particular end use. The manipulation of either the structural or regulatory genes of the starch biosynthetic enzymes could be used to alter the ratio of AM to AP, and change the properties of the starch, as well as the starch content in a plant (Preiss, 1992: Visser and Jacobsen, 1993). There is obviously great potential in many plants for the genetic manipulation of starch. It is possible that genetic manipulation might one day be performed on *Musa* germplasm to improve desirable quality characteristics of the starch. The genetic manipulation of the properties of a starch necessitates a knowledge of the synthesis of the starch. The following sections briefly describe starch biosynthesis in plants.

2.4 Starch Biosynthesis

The literature review has so far discussed *Musa* and starch, in the context of this research programme. The remainder of this review briefly describes the generally accepted theory of starch biosynthesis in plants, as reported in the literature. Some research has been done on starch in *Musa* fruits but, as far as is known, little research has been done on starch biosynthesis in *Musa*.

2.4.2 The Starch Biosynthetic Pathway

In starch biosynthesis there are three distinct processes comprising initiation (1), chain elongation (2) and branching (3):-

G-1-P $\rightarrow$ ADP-glucose $\rightarrow$ Amylose $\rightarrow$ Amylopectin

1 2 3

The major enzymes involved in the biosynthesis of starch are:

1, ADP-Glucose Pyrophosphorylase (ATP: α-D-glucose (-P Adenyl transferase), EC 2.7.7.27), which is involved in initiation;

2, Starch Synthase (ADP-glucose: 1,4-α-D-glucan-4-glycosyl-transferase, EC 2.4.1.21), for elongation of α-glucan molecules; and

3, Branching Enzyme (1,4-α-D-glucan-6-glycosyltransferase, EC 2.4.1.18), which is required for branching of the polyglucan chain to form AP molecules (and the subsequent formation of the starch granule) (cited by Smith, 1990a).

2.4.2.1 Supply of Substrate to Amyloplasts

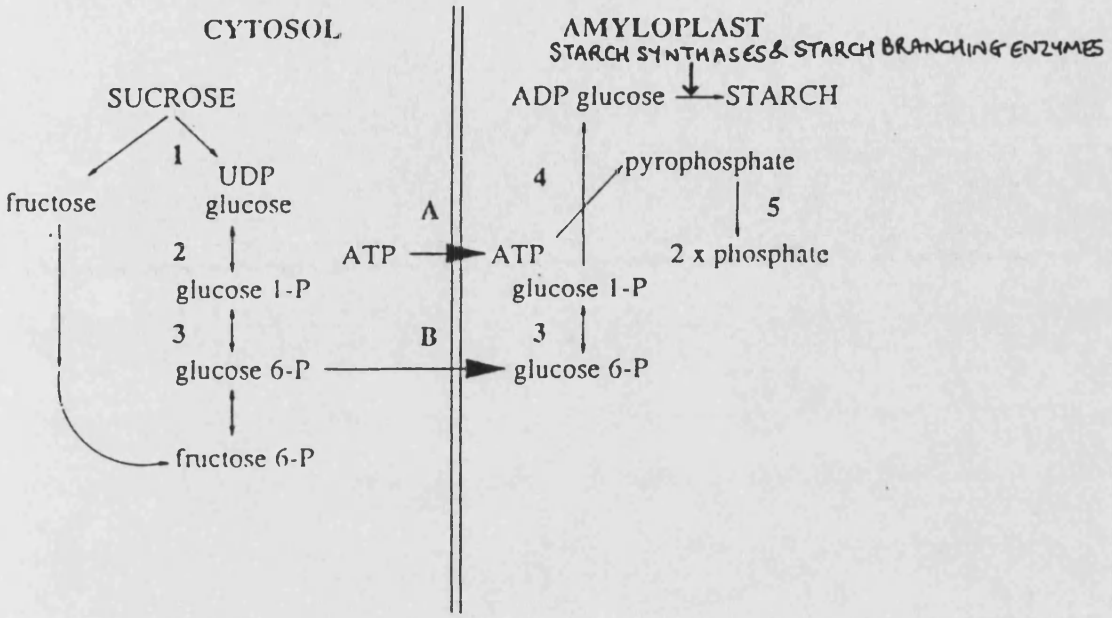
In the cytosol of pea cotyledonary cells, the supply of carbon to the amyloplasts is fed by alkaline invertase and sucrose synthase, which catalyse the breakdown of sucrose (Smith and Denyer, 1992). The probable metabolic pathway for the uptake of substrate by the amyloplast in shown in Figure 2.4.1. Uptake is probably in the form of G-6-P via a

translocator unique to these plastids (Smith and Denyer, 1992; Tyson and ap Rees, 1988). The mechanism may be a phosphate-exchange translocator which also recognises 3-phosphoglycerate and dihydroxyacetone phosphate, but not G-1-P or 2-phosphoglycerate (Smith and Martin, 1992). Thus, the hexose monophosphates, formed from successive cleavages of photosynthetically derived sucrose and UDP-glucose, are directly transported into the amyloplasts where they are converted into starch via the combined action of ADP-glucose pyrophosphorylase, starch synthase and branching enzyme (see Figure 2.4.1).

2.4.2.2 ADP-Glucose Pyrophosphorylase

The principal control point for starch synthesis is the conversion of glucose 1-phosphate (G-1-P) to ADP-glucose, by the exclusively plastidial enzyme ADP-glucose pyrophoshorylase (see Figure 2.4.1). Evidence of this was given when, in antisense transformation experiments, almost complete inhibition of the expression of this enzyme lead to starch-free potato tubers (Müller-Röber *et al.*, 1992).

Figure 2.4.1 Proposed Pathway of ADP-Glucose Synthesis in Developing Pea Embryos (Smith and Martin, 1992)



A, adenylate translocator

B, putative glucose 6-phosphate translocator

Enzymes catalysing the pathway:-

1, sucrose synthase

2, UDP-glucose pyrophosphorylase

3, phosphoglucomutase

4, ADP-glucose pyrophosphorylase

5, alkaline inorganic pyrophosphatase

2.4.2.3 Starch Synthases

Starch synthases build up linear chains of α-(1→4)-linked glucose residues by catalysing the addition of glucose from an ADP-glucose (substrate) molecule to the non-reducing end of pre-existing glucose chains or other primers. Starch synthase exists in plants both in the soluble fraction (stroma) of the plastid (soluble starch synthase, SSS), and as an enzyme tightly bound to the starch granules (granule-bound starch synthase, GBSS) (see Figure 2.4.2), though the precise roles of these two forms in starch synthesis are still under investigation (Smith, 1990a). The granule-bound starch synthase (GBSS) protein appears to produce AM exclusively (Smith and Denyer, 1992). Evidence to support this was given when antisense constructs of the GBSS gene were inserted into potatoes which produced plants with no AM, and little or no GBSS (Visser *et al.*, 1991). Additional evidence comes from work on maize. In maize the *Waxy* locus encodes the major granule-bound starch synthase (GBSS) protein. If the *Wx* protein is lacking, the mutants are *waxy* maize (which have the *wx* mutation) and no AM accumulates (Shannon and Garwood, 1984). A similar mutation, known as *amf*-1, is responsible for the absence of AM in potato starch (though this mutation does not occur naturally), as the *Amf* locus is the structural gene for GBSS (Van der Leij *et al.*, 1991).

Two isoforms of GBSS, termed GBSSI (59 kDa) and GBSSII (77 kDa), are associated with the starch granules in developing pea embryos. Pea embryo GBSSI (59 kDa) is almost exclusively granule-bound, and very similar in amino acid sequence to the *waxy* and *amf* gene products of cereal endosperm and potato, respectively (Dry *et al.*, 1992). Developing pea embryo starch GBSSII appears to be a novel type of granule-bound starch synthase, which carries a novel 203 amino acid domain at its N-terminus, and the C-terminal 60 kDa of the protein has a similar amino acid sequence to GBSSI and 'waxy' gene products (Dry *et al.*, 1992). Towards the end of pea embryo development, GBSSI represents about 80% of the total granule-bound protein, *i.e.* it is the major starch granule-

bound starch synthase (SGBSS) (Smith and Denyer, 1992). The molecular weights of the major SGBSSs in *Musa* starches, and their immunological cross-reactivity to antibodies raised to the same enzyme in pea starch, are investigated in Chapter 7.

The soluble starch synthases (SSSs) produce polymers which are substrates for AP via starch branching enzyme (SBE). It is not known how the product of GBSS (*i.e.* AM) is protected from branching. However, it is possible that the location of the enzyme on the surface of the granule renders its product physically or chemically unavailable to the branching enzymes (Smith and Denyer, 1992). In developing pea embryos, two starch synthases in the soluble fraction (stroma) of the amyloplast, SSSI (60 kDa) and SSSII (77 kDa), have very similar kinetic properties and are antigenically related to each other and to GBSSII, but not GBSSI (Denyer and Smith, 1992). Evidence suggests that the pea embryo 77 kDa soluble starch synthase (SSSII) is the same protein as GBSSII, and a protein resembling or identical to the 60 kDa soluble starch synthase occurs on the granule surface (cited by Smith and Denyer, 1992). Therefore, it has been suggested that both of the 'soluble' starch synthases could be present both in the soluble fraction and on the granule (see Figure 2.4.2). In maize, the soluble and granule-bound enzymes are quite distinct from each other (MacDonald and Preiss, 1985). In maize, the GBSSI and SSSI proteins are 61 kDa and 70 kDa, respectively, and both of the GBSSII and SSSII proteins are 93 kDa (Preiss, 1988). SSSI is immunologically distinct from GBSSI, II and SSSII (Preiss, 1988). In maize, it has been suggested that the soluble and granule-bound starch synthases are probably products of different genes, based on their physical, kinetic and immunological properties (Preiss, 1992).

The fact that it is difficult to recover starch synthase activity in developing pea embryo starch has lead to the suggestion that the enzyme becomes incorporated into the granule as it grows, and most of the GBSSI protein associated with purified granules is probably

more internal to the granule than on the granule surface (Smith and Denyer, 1992). This suggestion is illustrated in Figure 2.4.2.

2.4.2.4 Starch Branching Enzyme

AM and AP branches are made in the starch granule by starch branching enzyme. This enzyme cuts a short chain of glucosyl units (20 or less) from the non-reducing end of an α -(1→4)-linked chain, and then joins the cleavage product to the side of the same or an adjacent chain (*i.e.* inter- or intra-chain transfer), via an α-(1→6)-linkage to create a branch (Preiss, 1988).

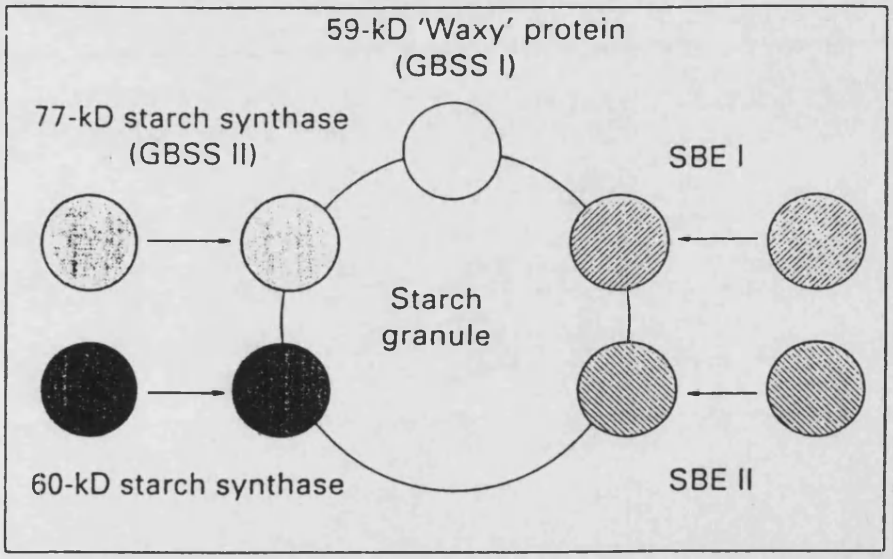
Various branching enzymes have been found in different starches. In maize endosperm, there are three forms of SBE, each 80 kDa in molecular mass (I, IIa and IIb). Immunological experiments have demonstrated that SBEI, SBEIIa and SBEIIb are all different proteins and products of different genes (Preiss, 1988).

Pea embryos have two isoforms of SBE, which are encoded by different genes, and their kinetic properties, patterns of expression, affinities for AM, and the solubilities they produce from a growing α-(1→4)-glucan chain are all different. SBEI and SBEII in developing pea embryos have molecular weights of approximately 112 kDa and 100 kDa, respectively, and both forms are in the soluble amyloplast fraction and bound to the starch granule (cited by Smith and Denyer, 1992) (see Figure 2.4.2).

The roles of SBEI and SBEII in granule synthesis and development are probably different, as, in developing pea embryo starch, the level of mRNA for SBEI peaks early in development, whereas that for SBEII peaks much later (Smith and Denyer, 1992). Branching probably occurs in the soluble phase at the granule periphery and BEs, like SSSs, become trapped within the granule structure as their product crystallises (see

Figure 2.4.2). How SBE isoforms interact with starch synthase isoforms to determine the structure of starch polymers is unknown.

Figure 2.4.2 Proposed Locations of Starch Synthases and Starch Branching Enzymes in the Amyloplasts of Developing Pea Embryos (Smith and Denyer, 1992)



2.5 Aims of this Research

Starch is known to be responsible for the cooking quality of plant products. Different *Musa* types are utilised for different end-uses, which is possibly related to the properties of the starch deposited within the pulp cells of the fruit. To test this hypothesis, the experiments described in this thesis are aimed towards widening the knowledge of some of the characteristics of starch granules which might be responsible for the cooking properties of the different *Musa* types. This research investigates:

- The starch contents of the flours produced from unripe and ripe *Musa* fruits.
- Physical, structural and molecular characteristics of starches of different *Musa* types.
- The cooking properties of *Musa* starches and flours.
- The relationship between the physical, structural and molecular characteristics of the starches of the different *Musa* types and their cooking properties.
- Characteristics of the major starch granule-bound starch synthase protein (the molecular weight and immunological cross-reactivity) of starches of different *Musa* types, as a preliminary experiment to future research on the starch biosynthetic enzymes in *Musa* fruits.

Chapter 3

General Materials and Methods

3.1 Plant Material and Storage Conditions

3.1.1 *Musa* Types

The *Musa* types (genome and sub-group) used in the experimental programme were dessert bananas (genome AAA, Cavendish), which had been grown in the Windward Islands, the Dominican Republic, and Jamaica; plantains (genome AAB, Horn) from the Dominican Republic and Colombia; and cooking bananas (genome ABB, Bluggoe) from Uganda and the Dominican Republic. The *Musa* fruits which were grown in the Windward Isles were supplied by Geest, Chippenham, U.K. All of the other *Musa* fruits were purchased from Spitalfields market, Leyton, London (see also Appendix Ia). The fruits had not been and were not artificially ripened in any way. When the fruits were purchased, it was estimated that approximately 14 days had elapsed since the fruits were harvested due to transportation and storage times. The *Musa* fruits were mature green and unripe on the day of purchase.

The *Musa* fruits were stored inside their cardboard box and polythene covering in a temperature-controlled environment at 14°C (which is the optimum temperature for the storage of bananas and plantains) at 80 ± 5% RH, from the time when they were purchased from the market until they were used for experimentation or sample preparation. The polythene covering inside the boxes and high humidity delayed ripening of the stored fruits (Thompson *et al.*, 1974). The fruits were randomly selected from the boxes when required. When the fruits were completely ripe (colour score 7 to 8, see section 3.2.1), they were discarded.

3.1.2 Reference Samples

The following samples were used in the experimental programme as comparatives or reference materials with the *Musa* flour and starch samples:

Reference Material	Supplier	Storage Conditions
Maize Starch Waxy Maize Starch ('Amioca Powder') Hylon V Starch Hylon VII Starch	National Starch and Chemical, Manchester, U.K.	Stored at room temperature.
Potato Starch Waxy Maize Starch	Unilever, Sharnbrook, Bedford, U.K.	Stored at room temperature.
Potato Starch Pea Starch Wheat Starch	John Innes Institute, Norwich, U.K.	Stored in a refrigerator at 1-5°C.

3.2 Methods

Within this section, the protocols of the techniques frequently used throughout the experimental programme are described.

3.2.1 Fruit Maturity

The disappearance or loss of peel green colour and the corresponding increase in yellowing of the peel during ripening are the obvious manifestations of the banana fruit. Peel yellowing during ripening is primarily due to chlorophyll degradation: the chlorophyll content decreases slowly with ripening (Palmer, 1971). The colour of banana peel is a widely used index of fruit maturity, as colour is a key characteristic of fresh produce and processed products. Peel colour charts have been developed to help standardise banana ratings for industry and research purposes (Von Loesecke, 1950). The colour of the banana peel was determined visually and classified according to a banana ripening guide

prepared for commercial use (from Chiquita Brands Inc., 1975), in which eight stages of peel colour were reproduced and translated to a numerical scale as follows:

1, the fruit is firm and completely green

2, green with traces of yellow

3, more green than yellow

4, more yellow than green

5, yellow with green tips

6, fully yellow

7, yellow with senescent spots

8, yellow, senescent spots coalesced.

In addition, as the exact age of the *Musa* fruits post-harvest was unknown, for each experiment, the number of days which had elapsed from the purchase of the fruits until processing (flour or starch isolation), or freezing (see below) was recorded. For ease of reference, throughout the experiments described in this thesis, when the fruits were designated as colour score index 1 to 3, the fruits were described as 'unripe', and when the fruits were designated as colour score index 6 or more, then the fruits were described as 'ripe'.

3.2.2 Isolation and Storage of Banana and Plantain Starch Granules

For the purposes of this research programme, starches were isolated from *Musa* fruits by a new method. Fingers of fruit were taken randomly from the box. The peel was either snapped off or cut off from the pulp of each finger by hand. *Musa* pulp (at least 100 g per starch isolation) was sliced into ~1 cm slices, along the length of the fruit. The ripening process was arrested by freezing the pulp with liquid nitrogen. The frozen pulp was either stored at -30°C until required for starch isolation, or the frozen pulp was used for starch isolation immediately. In the former case, the sample was labelled with the number of

days which had elapsed between the purchase of the fruits and the day on which the pulp was frozen. The frozen pulp was blended with 100 ml of isolation medium at 1°C (to minimise residual enzyme activity). The isolation medium contained 50 mM Tris HCl (pH 7.5), 10 mM EDTA, 10 mM $MgCl_2$, 0.75% (w/v) polyvinylpyrrolidone (PVP) of molecular weight 10,000, and was based on that used by Fishwick and Wright (1980), with modifications (Dr A. M. Smith, pers. comm.). PVP was included in the starch isolation medium to prevent browning of the blended pulp suspension by preventing oxidation of the polyphenols (see Hulme *et al.*, 1964a and b; Jones *et al.*, 1965; Walker and Hulme, 1965). The mixture was blended for 30 seconds, squeezed through muslin, and sieved through a 250 μm mesh Wilson sieve (Wilson Sieves, D. Wilson, Hucknall, Nottingham, U.K.) (to remove fibrous material and other broken cell components), and an 180 μm mesh Wilson sieve, into a flask containing 50 ml of extraction medium (at 1°C). A further 100 ml of isolation medium (at 1°C) was used to encourage the drainage of the material through the sieves. The material was allowed to drain for at least 15 min. The filtered material was further passed through a 90 μm mesh Wilson sieve, and spun in a refrigerated centrifuge for 5 min at 3,300 rpm (Chilspin, MSE, Fisons, U.K.). The pellet of starch granules was washed and centrifuged for 5 min at 3,300 rpm (Chilspin) four times with 50 mM Tris-HCl (pH 7.5) at 1°C, and then washed and centrifuged for 5 min at 3,300 rpm (Chilspin) four times with distilled water to remove solutes. The suspension of starch granules in distilled water was air-dried in shallow metal dishes overnight in an oven at 30°C (Gallenkamp 300 Plus Series), prior to storage at 1-5°C in 60 ml screw-cap glass jars, which were sealed with PVC tape. The starch isolation procedure was not quantitative, and was only intended to provide enough starch for analysis within the experimental programme. The yield of starch granules recovered form a routine starch isolation, using 100-200 g of frozen *Musa* pulp, was between 5-10 g.

3.2.3 Production and Storage of *Musa* Flour

About 10-20 fingers were taken randomly from each box of fruit. The peel from each finger was snapped or cut off from the pulp by hand. The pulp was then chopped into small chunks which were frozen immediately with liquid nitrogen. Freezing arrested the ripening process and the frozen pulp was either stored at -30°C until required, or it was processed into flour straight away. The frozen pulp was arranged in shallow layers on dishes or trays which were placed in a freeze-drier made by Chemlab Instruments Ltd., using an Edwards High Vacuum Pump, Model E2M5, for about 3 days, when the pulp was completely dried.

The dried pulp was weighed and then blended in a Waring blender for a few seconds, to make flour. The blended material was then sieved through a 425 μm mesh laboratory test sieve. *Musa* flour was stored in 1 litre screw-cap glass jars, which were sealed with PVC tape, at 1-5°C. Ukhun and Ukpebor (1991) found that this was an optimum storage temperature for their instant plantain flours.

3.2.4 Determination of the Moisture Contents of the Starches and Flours (dry weight method)

The moisture contents of all of the *Musa* starch and flour samples was determined prior to all analyses. Approximately 2 g of sample (starch or flour) was put into a metal dish (with lid) of known weight (w_1) and the combined weights recorded (w_2). This was oven-dried (Gallenkamp 300 Plus Series) at 106°C for 4 h. After the drying period, the dishes and lids were allowed to cool in desiccators before weighing (w_3). The percentage moisture content of the sample was calculated as follows:

$$\% \text{ moisture content} = \frac{w_2 - w_3}{w_2 - w_1} \times 100$$

3.2.5 Determination of the Starch Contents of the *Musa* Flours

It was necessary to remove the alcohol soluble solids from the *Musa* flours prior to acid hydrolysis and the determination of their starch contents.

3.2.5.1 Preparation of Alcohol Insoluble Solids (Extraction of Soluble Sugars)

Analyses were carried out in triplicate. A known weight of sample of known moisture content (section 3.2.4), filling no more than two thirds of a Whatman cellulose extraction thimble (30 mm x 80 mm) (Whatman International Ltd., Maidstone, Kent, U.K.), was plugged with non-absorbent cotton wool. The thimble (together with its contents and cotton wool) was then re-weighed. The thimbles were refluxed with 200 ml of 80% ethanol (in a 250 ml flat bottomed flask also containing 3 x 3 mm glass balls) for 2 h using Soxhlet equipment (Quickfit). After this time the apparatus was allowed to cool before removing the thimbles containing the alcohol insoluble solids. The liquid left in the extraction equipment was a solution of ethanol soluble material. This was discarded. The ethanol-refluxed samples were dried overnight in an oven (Gallenkamp 300 Plus Series) at 60°C. The weight of the alcohol insoluble material was recorded. The thimbles were stored in a desiccator until required for further analyses (*e.g.* moisture content determinations and starch content determinations). The starch contents of the alcohol insoluble solids were determined using the glucose oxidase method. However, the starch initially had to be hydrolysed.

3.2.5.2 Acid Hydrolysis

It was necessary to acid hydrolyse the flour samples to break up the starch granules and starch polymers. The hydrolysis of the flour was carried out according to the method reported by Rickard and Behn (1987). Samples (200 mg) were refluxed with 0.7 M HCl (110 ml) in 250 ml round-bottomed flasks (also containing 3 x 3 mm glass balls) for 150 min on a heating mantle. After hydrolysing, the hydrolysates were cooled to room

temperature, neutralised to pH 7 with 3 M sodium hydroxide, and made up to 500 ml in volumetric flasks. An aliquot of each sample (50 ml) was filtered through glass micro-fibre filter paper (Whatman GF/A) before glucose determination.

3.2.5.3 Glucose Oxidase Method

Several methods were tested for the determination of the starch content of the *Musa* flours. The methods comprised the phenol-sulfuric acid colorimetric method (Hodge and Hofreiter, 1962a), the anthrone colorimetric method (Hodge and Hofreiter, 1962b), a ferricyanide reduction method (as described by Rickard and Behn, 1987), and the glucose oxidase method (described below). The glucose oxidase method was found to be the only successful method for determining the starch content of the *Musa* flours as this assay was specific for glucose, and was not affected by the presence of pigments in the acid-hydrolysed flour samples.

The acid hydrolysed flour samples were assayed for their glucose contents as follows. The glucose content of each filtered starch hydrolysate was determined in triplicate by the glucose oxidase method using the Boehringer test kit (No. 124 036). Aliquots of sample (0.1 ml) and Boehringer buffer/enzyme/chromogen reagent (5 ml) were mixed and incubated at room temperature (20-25°C) for 25 min. The absorbance was read at 420 nm on a Philips PU 8620 UV/VIS/NIR Spectrophotometer against a reagent blank prepared from water (0.1 ml) and reagent (5 ml). A standard curve was prepared using glucose (50-500 mg litre^{-1}). Starch concentration was calculated as glucose concentration x 0.9 (adjusted for free sugar content). See Appendix Id for the calculations which were used in determining the starch contents of the *Musa* flours.

3.2.6 Ethanol-Reflux Method (Defatting)

All of the *Musa* flour samples and the reference maize and waxy maize starches were ethanol-refluxed prior to mixing with water for the Brabender Viscoamylograph and Rapid Viscoanalyser experiments (see Chapter 6). This procedure was employed as otherwise the *Musa* flour suspensions were lumpy and immiscible in water.

The procedure used was the same as that described in section 3.2.5.1 for obtaining alcohol insoluble solids. After drying the alcohol insoluble solids at 60°C overnight, the dry alcohol insoluble solids were put into a plastic bag and rolled with a rolling pin to break down any lumps prior to sieving through a 425 μm mesh sieve. The sieved ethanol-refluxed samples were stored at 1-5°C in 1 litre screw-cap glass jars, which were sealed with PVC tape, until required for analysis (*e.g.* moisture content determinations and use in the Brabender Viscoamylograph and Rapid Viscoanalyser).

Chapter 4

Physical Characteristics of *Musa* Starch Granules

4.1 Introduction

Some of the characteristics that most markedly affect the behaviour of starch and allow differentiation between starches of different origin are their particle sizes, particle size distributions, and the shapes of the granules (Rašper, 1971). In this chapter, the physical characteristics (shapes and sizes) of starch granules isolated from different *Musa* types were examined. The effect of the starch isolation procedure on the physical appearance of the starch granules was also investigated.

4.1.1 Physical Appearances of Dessert Banana Starch Granules at Different Stages of the Starch Isolation Procedure

In order to interpret the results of the analysis of starch characteristics, it is important to determine to what extent the isolation procedure may affect the properties of the starch granules. With this consideration, Transmission Electron Microscopy (TEM) was undertaken to observe the two-dimensional structures of starch granules isolated from mature green unripe dessert bananas throughout four different stages of the starch isolation procedure. The intactness of the amyloplast membrane during the starch isolation procedure could also be studied. The amyloplast membrane is a fragile double layer which encloses the starch granule (section 2.3.1) and the enzymes of starch biosynthesis (section 2.4).

4.1.2 Physical Appearances and Sizes of the Starch Granules

Scanning Electron Microscopy (SEM) is often used for characterising the morphology of plant and animal tissues beyond the resolution limit of the optical light microscope. In this experiment, the SEM was used to observe the three-dimensional shapes of the starch granules in sections of mature green unripe dessert banana pulp.

The polarised optical light microscope (OLM) was used to observe the sizes and shapes of starch granules which had been isolated from different *Musa* types. Other observed physical features of the starch granules were the appearance of the starches when viewed between crossed polars, the position of the hilum, and the presence of striations (or 'growth rings') in the granules.

The Coulter Counter technique is considered to be more accurate for particle size determination than other techniques, especially with coarser materials such as flour and starch (cited by Rašper, 1971). The Coulter Counter® Model TAII determines the numbers and sizes of particles suspended in a conductive liquid, by monitoring the electrical current between two electrodes immersed in the conductive liquid on either side of a small aperture through which a suspension of the particles is forced to flow. As each particle passes through the aperture, it changes the resistance between the electrodes and produces an electrical pulse of short duration whose amplitude is essentially proportional to the particle diameter. The series of pulses are electronically amplified, sized and counted, and from the derived data, the particle size distribution of the particles in suspension may be determined. The particle size distributions and geometric mean particle sizes (*i.e.* diameters) of starches which had been isolated from different *Musa* types, were determined using the Coulter Counter. Potato starch, waxy maize starch and maize starch were used as reference samples.

4.2 Materials and Methods

4.2.1 Transmission Electron Microscopy

Starch from mature green unripe dessert bananas from the Windward Isles was taken for transmission electron microscopy analysis at four different stages during the starch isolation procedure, according to section 3.2.2:

1, intact pulp
2, blended pulp in the isolation medium
3, sieved pulp suspension, and
4, freeze-dried starch granules (instead of air-dried starch granules as in section 3.2.2, for speed of the isolation procedure).

TEM Sample Preparation of Banana Pulp and Banana Starch Granules

All TEM sample preparation was performed in a fume cupboard. Samples were taken at four different stages of the starch isolation procedure and prefixed with 4% paraformaldehyde, 2.5% glutaraldehyde and 0.75% acrolein in 100 mM sodium cacodylate pH 7.2. Cubes (1 mm^3) of banana pulp (Stage 1 of the starch isolation procedure) were left to stand overnight in the fixative at 4°C. Aliquots of the pulp suspension, taken after the blending and sieving stages (Stages 2 and 3, respectively) during the starch isolation procedure, were spun in a refrigerated centrifuge for 5 min at 3,300 rpm (Chilspin, MSE, Fisons, U.K.), and the Tris-HCl extraction medium (section 3.2.2) removed before re-suspending the pellet in fixative for 2 h at 4°C. In the final step of the starch isolation procedure, after the starch granules had been washed with distilled water (as in section 3.2.2), the pellet of starch granules was freeze-dried (Stage 4) (at -40°C) for a few hours until dry, using an Edwards high vacuum pump. Freeze-dried starch granules were suspended in fixative for 2 h at 4°C.

All fixed samples were washed with 100 mM sodium cacodylate pH 7.2, for 3 x 15 min. The post-fix was 100 mM sodium cacodylate and 2% OsO_4 for 120 min, followed by washing with 100 mM sodium cacodylate, pH 7.2, for 3 x 15 min. Samples were then taken through a series of dehydration steps of 30, 50, 70, 95, and 100% ethanol, for 30 min each, at room temperature.

Samples were then put into 2% agar and cut into blocks of material (2-3 mm^3). The embedding was carried out in epoxy resin. The embedded samples were put into 1:1 100% ethanol: propylene oxide (30 min), 100% propylene oxide (30 min), 3:1 propylene oxide: premix resin (1 h), 1:1 propylene oxide: premix resin (overnight), and 1:3 propylene oxide: premix resin (1 h). Samples were put into 100% premix resin (2 h). Then the samples were put into beam capsules at 60°C for approximately 76 h.

As a preliminary check, for the presence of starch granules and their suitability for observation with the TEM, semi-thin sections (~7.5 μm) were taken with a Om U3 ultramicrotome (C. Reichert, Austria) using glass knives. The sections were stained with toluidine blue and viewed under an OLM. Then ultra-thin sections (60-100 nm) were made and picked up on copper grids. They were then stained with 2% uranyl acetate (10 min), washed with boiled distilled water (5 min), then stained with Reynolds reagent (lead citrate) for 10 min. Some NaOH on filter paper was put inside the same closed container as the Reynolds reagent staining step to absorb any CO_2. Then sections were washed well with boiled distilled water (5 min). The copper grids were dried on filter paper. Sections were viewed with a Jeol Transmission Electron Microscope 1200 EX at 80 kV.

4.2.2 Scanning Electron Microscopy

Cubes of pulp were taken from mature green unripe dessert bananas which had been grown in the Windward Isles.

SEM Sample Preparation of Banana Pulp

Using an Oxford Instruments Hexland CT100 Cryo System, attached to the Jeol JSM-35C Scanning Electron Microscope, it was not necessary to fix or treat the samples in any way other than to plunge them into liquid nitrogen before inserting into the pre-chamber of the SEM. Each cube of *Musa* pulp was given a gold sputter-coating prior to viewing the sample in the SEM and taking photographs.

4.2.3 Optical Light Microscopy

Starches were isolated, according to section 3.2.2, from unripe dessert bananas which had been grown in the Windward Isles, unripe Dominican Republic dessert bananas (from day 1 after purchase of the fruits), unripe Jamaican dessert bananas (from day 1), ripe Colombian plantains (from day 18), unripe Dominican Republic cooking bananas (from day 3), and semi-ripe Ugandan cooking bananas. Potato starch, waxy maize starch and maize starch were used as reference starches.

To observe the starch granules under the OLM, the starches were suspended in either distilled water (Plates 4.3.6 and 4.3.11), or a mixture of 1:2 (v/v) Anal R glycerol-water (distilled) (Plates 4.3.7 to 4.3.10, and 4.3.12 to 4.3.14). One drop of the solution was put onto a clean microscope slide and a 'pin-head' of starch was mixed into this drop. Then the cover-slip was carefully placed on the starch suspension. The cover-slips were sealed with ordinary nail varnish (to minimise the movement of granules in the field). The starch granules were viewed with a 40 x objective using an Olympus BHS (Plates 4.3.6 and 4.3.11) and a Leitz Diaplan Optical Microscope with a polarising attachment (Plates 4.3.7 to 4.3.10 and 4.3.12 to 4.3.14). The starch granules were photographed with a Ricoh KR-10 M camera. A lambda plate was used with crossed polarising filters which produced the colour effect (Plates 4.3.7 to 4.3.10 and 4.3.12 to 4.3.14). A photograph of

a graticule (100 x 0.01 = 1 mm. Graticules Ltd., Tonbridge, Kent, U.K.) was used to determine the magnification levels of the photographs of the starch granules.

4.2.4 Coulter Counter

Starches were isolated, according to section 3.2.2, from unripe Dominican Republic dessert banana (from day 1 after purchase of the fruits), unripe and ripe Jamaican dessert banana (from day 1 and day 20, respectively), unripe and ripe Colombian plantain (from day 1 and day 18, respectively), and unripe Dominican Republic cooking banana (from day 3). Potato starch, waxy maize starch and maize starch were used as reference starches.

The electrolyte, supplied by Coulter Counter Euro Diagnostics, GMBH, U.K., was composed of NaCl (7.9 g/l), disodium hydrogen orthophosphate (1.9 g/l), EDTA disodium salt (0.4 g/l), potassium chloride (0.4 g/l), sodium dihydrogen orthophosphate (0.2 g/l) and sodium fluoride (0.3 g/l).

The Coulter Counter® Model TAII was fitted with a 200 μm orifice tube. The instrument was calibrated with a latex suspension of 20 μm latex particles (Calibration Standard, PDVB Latex, Coulter Electronics Ltd., Luton, Beds., U.K.) on Channels 9 and 10.

To 45 mg of each starch sample was added 0.8 ml of electrolyte. From this was taken two drops which were added to the Coulter Counter beaker filled to three-quarters of its volume capacity with electrolyte. A suspension concentration of between two and five percent was used for each starch sample. Each starch sample was tested in duplicate.

As there was electrical noise in Channels 1 to 4, readings were taken from Channel 5 through to Channel 15. Each reading was expressed as a percentage of the total number

of counts. The raw data was corrected for background noise. Plots were made of the percentage Frequency versus Channel Number. As the instrument had been calibrated, a Particle Size scale was prepared to plot the % Frequency versus the Particle Diameter (μm) for each starch sample. From the size distribution data, the geometric mean particle size, M_g, and the geometric standard deviation, σ_g, were calculated, based on the assumption that the granule sizes were distributed as a log normal distribution. Headley and Pfost (1968) and Rašper (1971) also used this approach for their calculations. Thus,

$$\ln S_{gw} = \left[\frac{\sum (W_i (\ln d_i - \ln d_{gw})^2)}{\sum W_i} \right]^{0.5}$$

and, the geometric mean particle size (M_g) by the relationship:

$$\ln d_{gw} = \frac{\sum (W_i . \ln d_i)}{\sum W_i}$$

where,

d_{gw} = Geometric Mean Particle Size by weight or volume

S_{gw} = Standard Deviation by weight

d_i = Geometric Mean Particle Size (or diameter) for the i^{th} interval

W_i = Weight of Material in the i^{th} interval

4.3 Results and Discussion

4.3.1 Physical Appearances of the Dessert Banana Starch Granules at Different Stages of the Starch Isolation Procedure

During the TEM sample preparation of the cubes of banana pulp, the cytoplasmic material shrunk considerably. This suggested that perhaps such a high fixative concentration should not have been used. Previously, a fixative using 2.5% glutaraldehyde in Tris-HCl, pH 7.5, and L. R. White resin was used. However, this fixing procedure was unsuccessful, as the samples did not thin-section properly, due to water constantly absorbing onto the face of the block, and the resin was also flaky.

According to Morrison and Karkalas (1990), it is unrealistic to rule out artifacts in TEM experiments because of the high fixative concentrations used and the lengthy TEM preparation procedures. Nevertheless, amyloplast membranes were observed around the starch granules of the mature green unripe dessert banana pulp sample at Stage 1 (Plate 4.3.1). The amyloplast membranes were removed from the starch granules at the blending stage (Plate 4.3.2), which was evidence of the fragility of these plastids in *Musa* pulp. The isolation procedure must be delicately performed if amyloplasts are to be isolated in their entirety (*e.g.* for research on starch metabolism across these membranes). However, for the purposes of this research programme it was not necessary to isolate intact amyloplast membranes.

In the intact pulp cells of a mature green unripe banana (Plate 4.3.1), the cells were packed with starch granules and amyloplast (double) membranes could be seen around each starch granule. The sectional length of the largest starch granule in the TEM photograph was 11.75 μm. The cytoplasm had shrunk considerably in each cell, presumably due to cell dehydration caused by the high concentration of fixative used. Mitochondria and other organelles (*e.g.* ribosomes and endoplasmic reticulum) were seen

to be localised in the cytoplasm around the amyloplast membranes enclosing the starch granules. The cell walls were intact, though in some places along the middle lamella the cells had pulled away from each other where each cell wall had been dragged-in towards its own cell due to dehydration of the cells.

In the TEM photograph of the blended pulp, the pulp cells had been broken apart and broken open, and cellular debris could be seen (Plate 4.3.2). The surfaces of many of the starch granules were shadowed, which was probably due to fragments of cell debris sticking to the starch granules. The starch granules were also embedded in regions of cellular debris. The sectional length of the starch granule in the photograph was 13.7 μm. There did not appear to be any amyloplast membranes left intact around the starch granules at this stage in the starch isolation procedure. The blending step probably broke open the amyloplast membranes at a particular spot of weakness, causing them to shrivel-up and pull away from around the dense starch granules.

The TEM photograph of the sieved pulp suspension showed starch granules which were covered in dark patches and there was an uneven fuzziness around the granules (Plate 4.3.3). This 'halo' effect was probably due to the lead staining of the osmium-reacted areas (*i.e.* membrane lipids in the cellular debris). Cellular debris was stuck onto the surface and around the starch granules. The sieving step involved using a number of sieves of decreasing mesh size (from 250 to 90 μm) (section 3.2.2). Therefore, most of the cellular debris in the blended pulp suspension had been removed. The sectional lengths of the starch granules, in Plate 4.3.3, were 11.8 μm and 8.9 μm.

The shapes of the freeze-dried starch granules were varied (Plate 4.3.4). Some of the grains were surrounded by an uneven blurred area. This was possibly due to the TEM

sample preparation (staining), or small amounts of cellular debris sticking to the starch granules. The sectional length of the starch granule, in Plate 4.3.4,.was 11.2 μm.

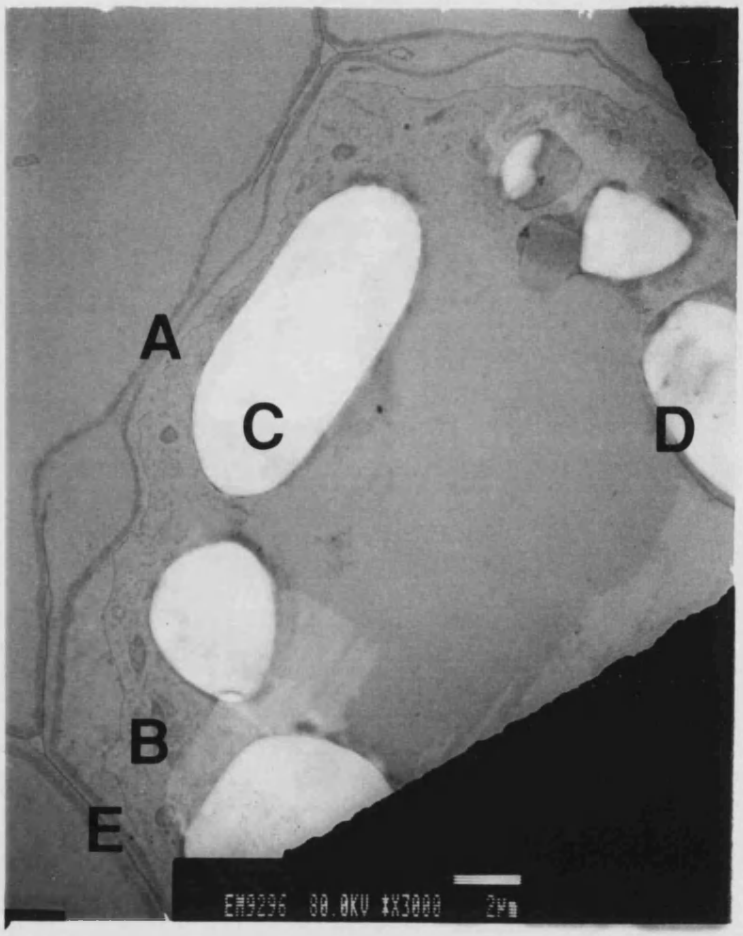


Plate 4.3.1 Transmission Electron Micrograph of a Mature Green Unripe Dessert Banana Pulp Section (Stage 1)

Key:-

A, Cell Wall

B, Mitochondrion

C, Starch Granule

D, Amyloplast Membrane

E, Middle Lamella

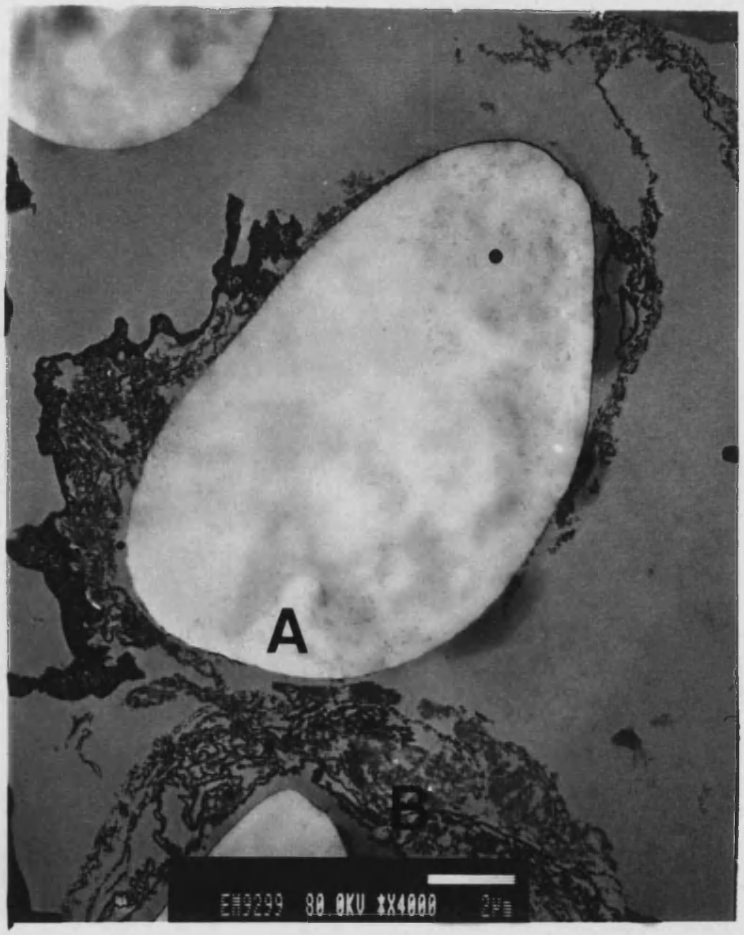


Plate 4.3.2 Transmission Electron Micrograph of a Mature Green Unripe Dessert Banana Blended Pulp Section (Stage 2)

Key:-

A, Starch Granule

B, Cellular Debris

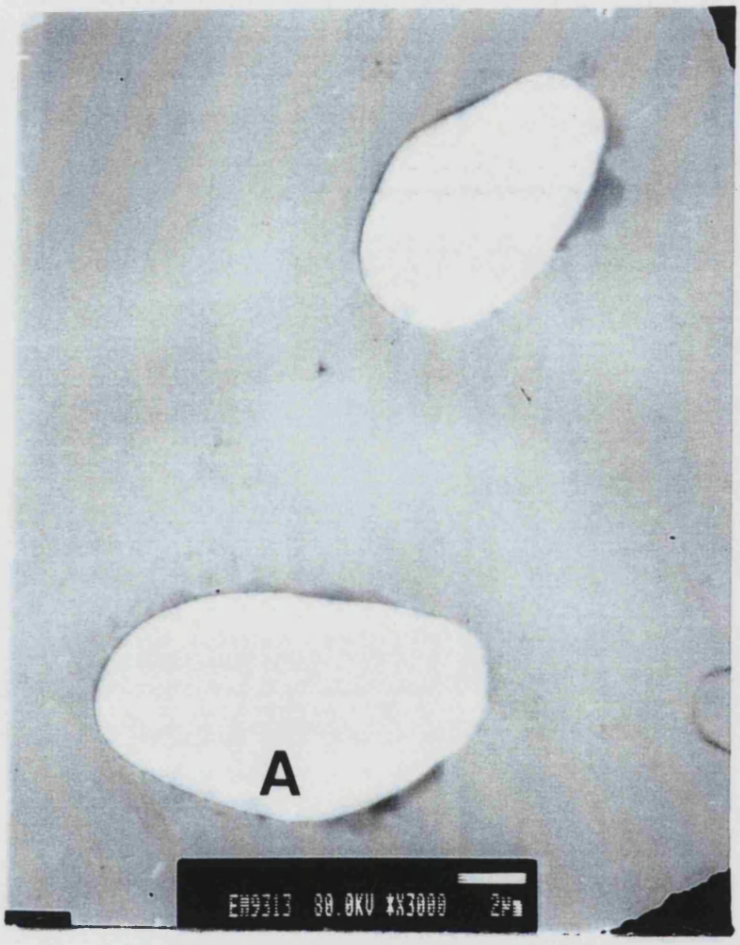


Plate 4.3.3 Transmission Electron Micrograph of a Mature Green Unripe Dessert Banana Sieved Pulp Suspension Section (Stage 3)

Key:-

A, Starch Granule

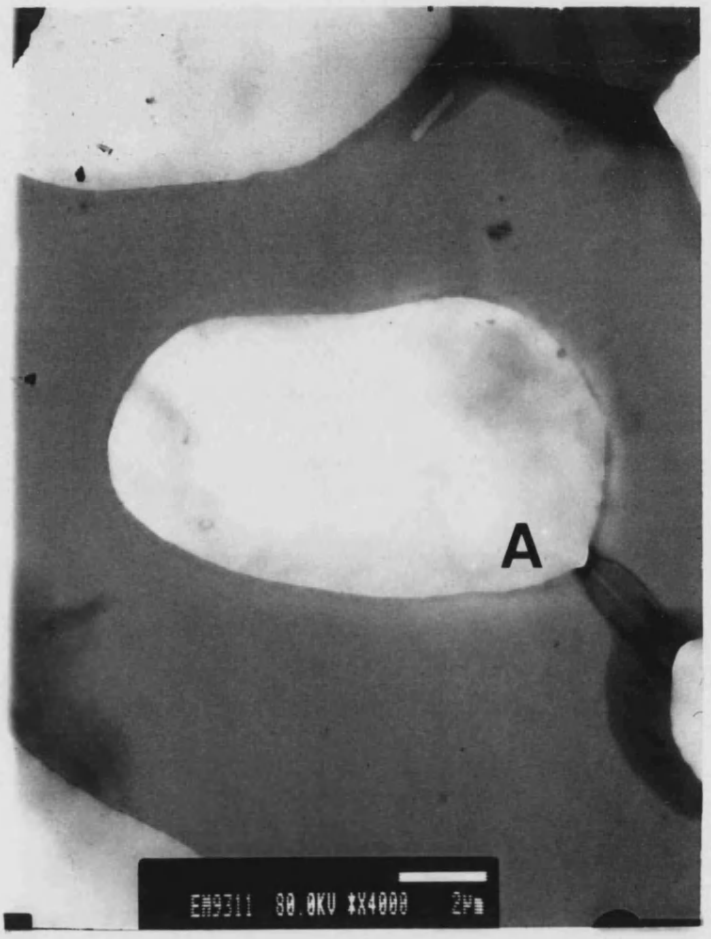


Plate 4.3.4 Transmission Electron Micrograph of a Mature Green Unripe Dessert Banana Freeze-Dried Starch Granule Section (Stage 4)

Key:-

A, Starch Granule

4.3.2 Physical Appearances and Sizes of the Starch Granules

As seen in Plate 4.3.5, when viewed under the scanning electron microscope, the unripe dessert banana pulp cells were packed with large irregularly-shaped starch granules, which were predominantly spheroid and elongated. The largest elongated starch granule is 48 μm in length and 18 μm in width The surfaces of the starch granules in the photograph are smooth, which suggests that the starch granules had not undergone any significant starch degradation and that the banana used in the experiment was mature but unripe (see Kayisu *et al.*, 1981). Kayisu *et al.* (1981) reported similar physical dimensions for dessert banana granules to those in Plate 4.3.5, of 15-40 μm for the spheroid type, and their elongated granules were 7-25 μm in width and 20-50 μm in length.

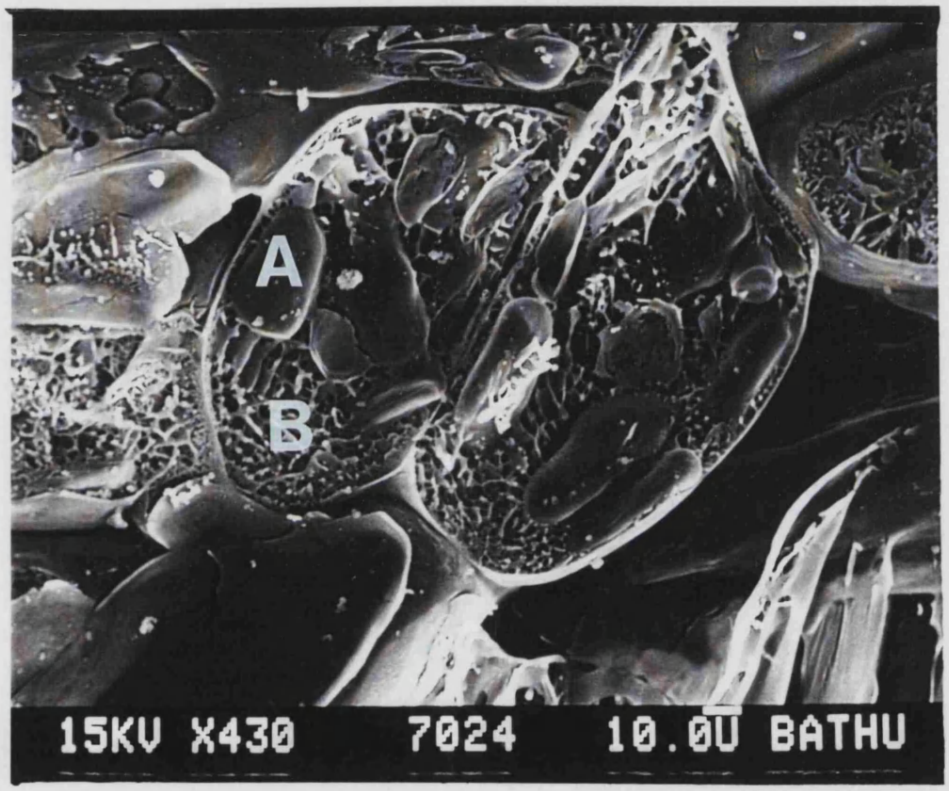


Plate 4.3.5 Scanning Electron Micrograph of Mature Green Unripe Dessert Banana Pulp Cells

Key:-

A, Starch Granule

B, Pulp Cell

In the optical light microscopy photographs there were no observable differences in the physical appearances of the starch granules of the same *Musa* type whether the starches were isolated from unripe or ripe fruits. Therefore, only one OLM photograph is shown of starch which was isolated from a ripe *Musa* type, *i.e.* plantain.

In the OLM photographs all of the *Musa* starch granules were oyster-shell in shape, irrespective of the *Musa* type, or country of origin (Table 4.3.1). However, the plantain and cooking banana starch granules appeared to be slightly more elongated and ovoid than those of the dessert banana. The potato starch granules were also oyster-shell in shape, whereas the starch granules of the two cereal starches were irregular in shape (Table 4.3.1). As the position of the hilum in the starch granules of all of the *Musa* starches and potato starch was eccentric, this would suggest an uneven deposition of the layers of the starch polymers during starch biosynthesis in both the *Musa* starch granules and the potato starch granules. In contrast, the centrally positioned hila in the two cereal starches suggested an even deposition of the layers of the starch polymers during starch biosynthesis. In all of the *Musa* starch granules, and in the potato starch granules, concentric layers of microfibrils ('growth rings') were observed which radiated outwards from the hilum (Plates 4.3.6 to 4.3.12). However, these growth rings (or striations) were not seen in the maize starch granules and waxy maize starch granules (Plates 4.3.13 and 4.3.14). All of the starch samples were birefringent and exhibited a 'Maltese Cross' when viewed between crossed polars (Plates 4.3.6 to 4.3.14), with the arms of the cross intersecting at the hilum (see section 2.3.3.2). The arms of the polarisation cross coincided with the chain direction of the starch, which was perpendicular to the growth rings. The sizes of the starch granules of the different *Musa* starches were similar. The potato starch granules were also similar in size to those of the *Musa* starches, though the maize starch granules and waxy maize starch granules were much smaller (Table 4.3.1).

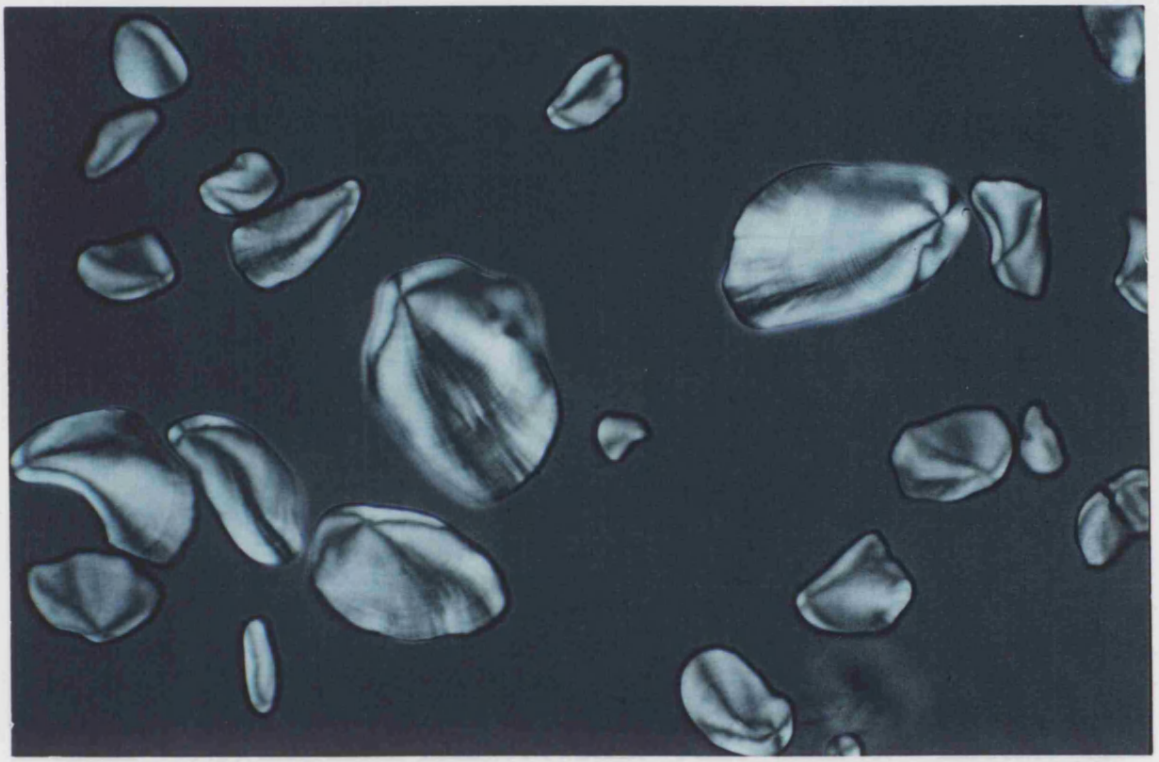


Plate 4.3.6 Polarised Optical Light Micrograph of Unripe Dessert Banana Starch Granules from the Windward Isles (Magnification x 590)

Plate 4.3.7 Polarised Optical Light Micrograph of Unripe Dominican Republic Dessert Banana Starch Granules (day 1) (With a lambda plate. Magnification x 600)

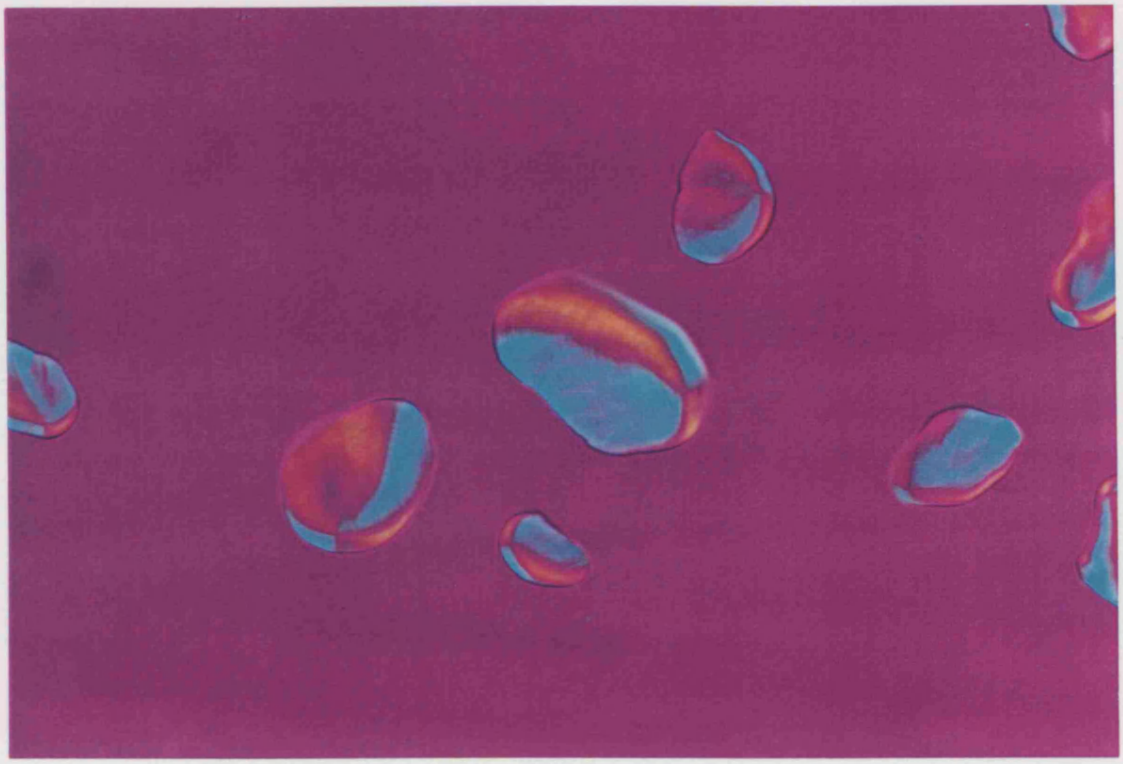


Plate 4.3.8 Polarised Optical Light Micrograph of Unripe Jamaican Dessert Banana Starch Granules (day 1) (With a lambda plate. Magnification x 600)

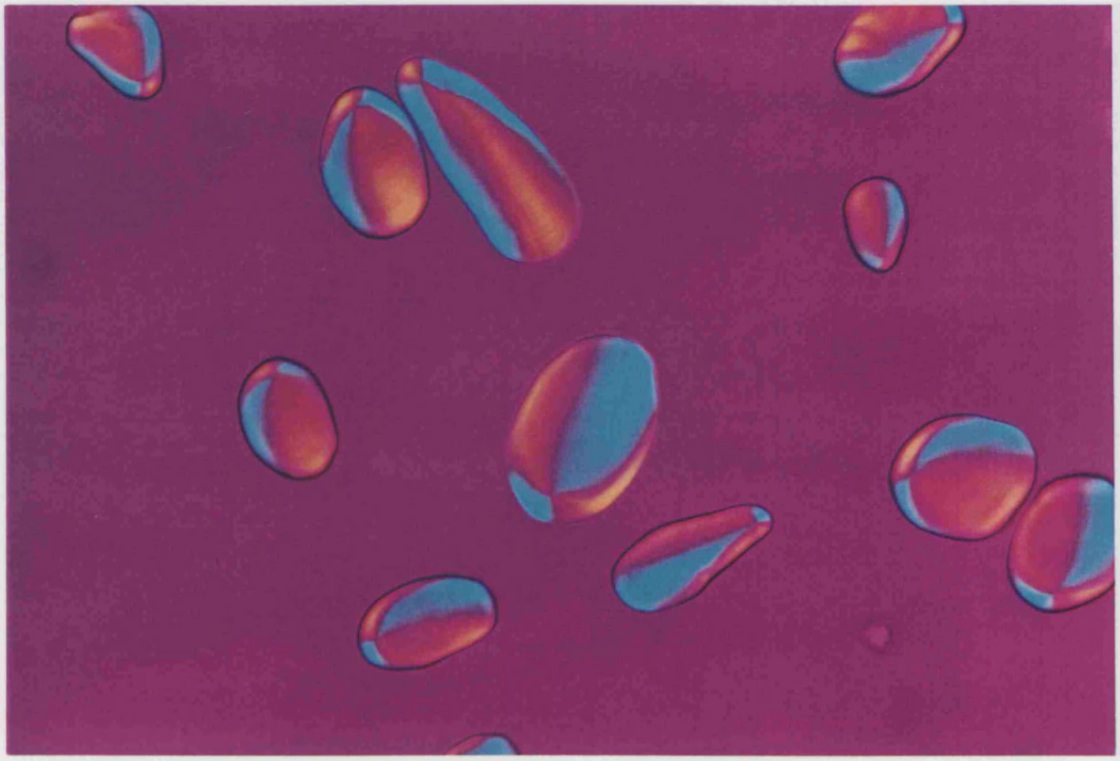


Plate 4.3.9 Polarised Optical Light Micrograph of Ripe Colombian Plantain Starch Granules (day 18) (With a lambda plate. Magnification x 600)

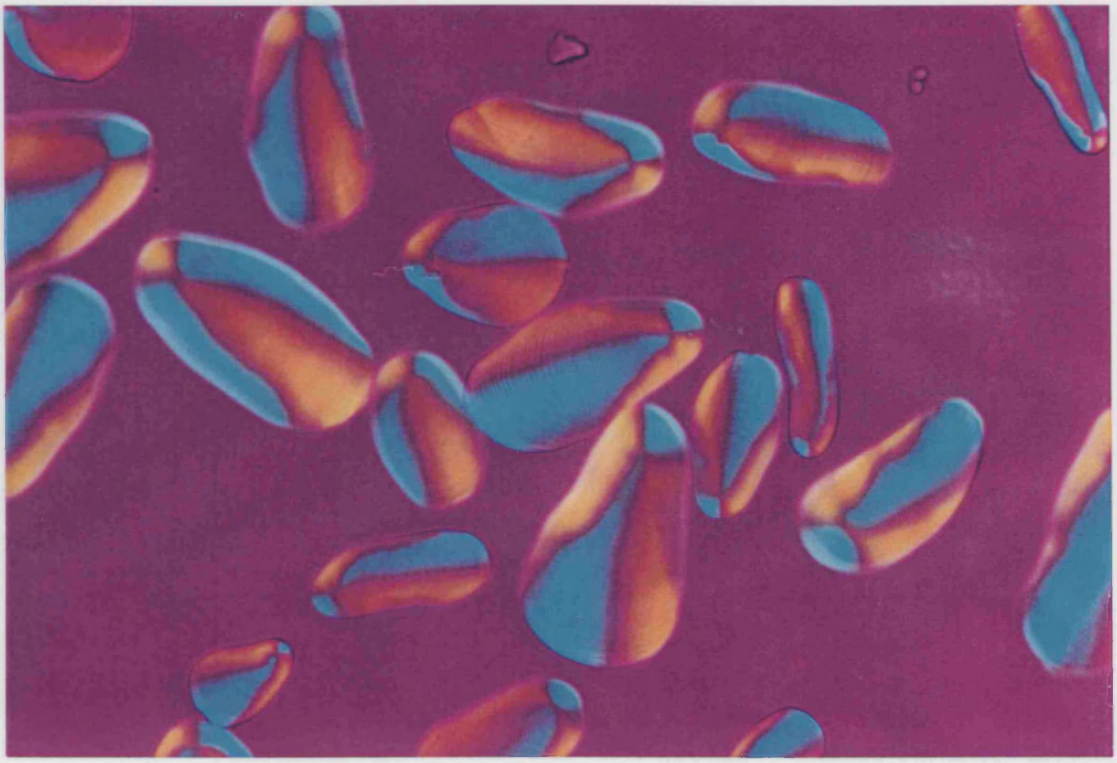


Plate 4.3.10 Polarised Optical Light Micrograph of Unripe Dominican Republic Cooking Banana Starch Granules (day 3) (With a lambda plate. Magnification x 600)

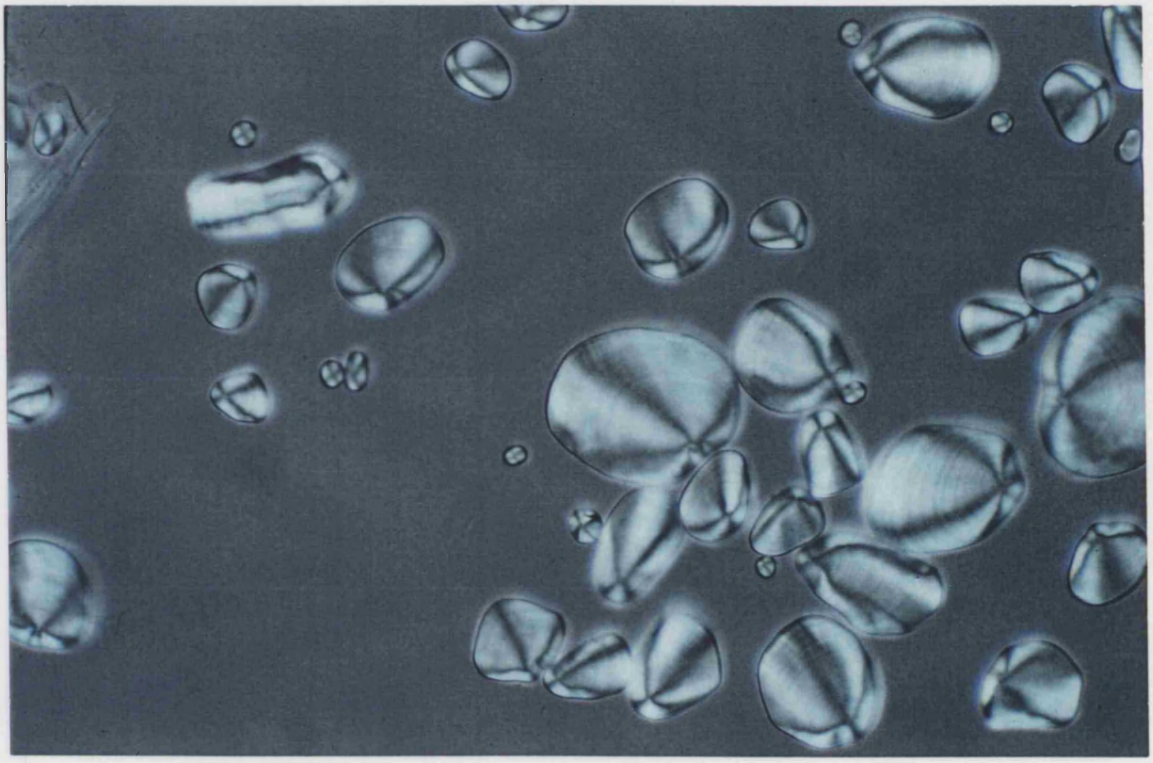


Plate 4.3.11 Polarised Optical Light Micrograph of Unripe Ugandan Cooking Banana Starch Granules (Magnification x 590)

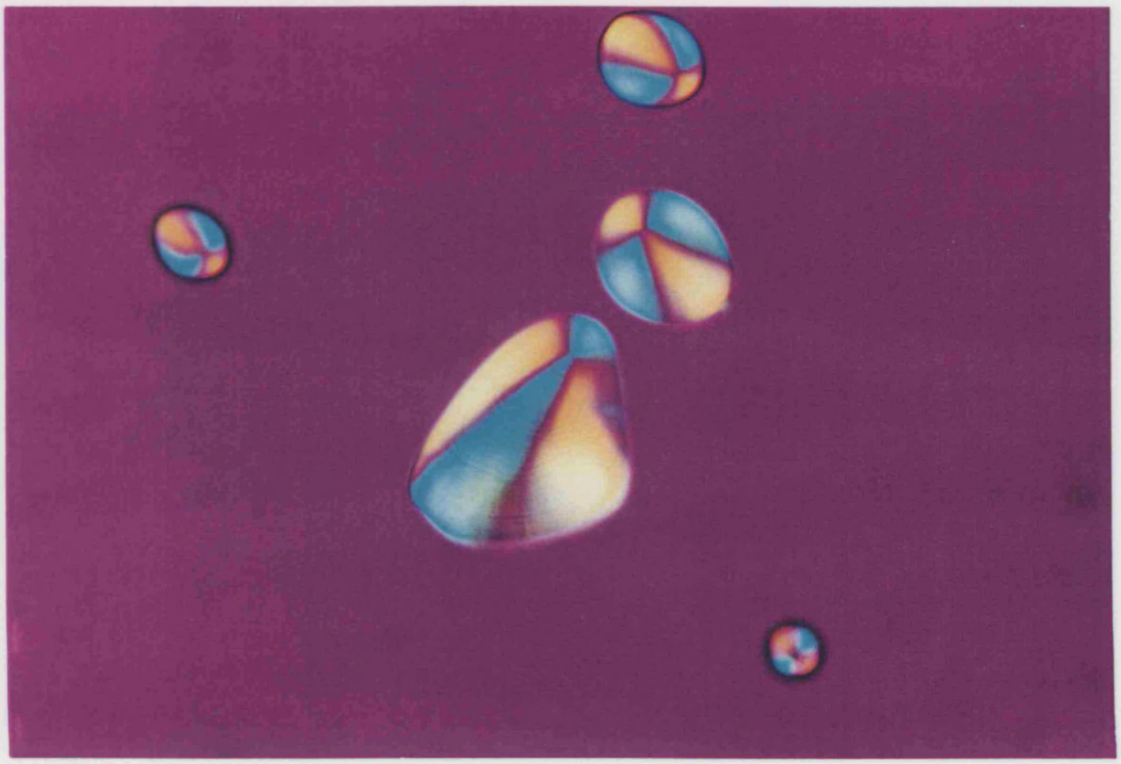


Plate 4.3.12 Polarised Optical Light Micrograph of Potato Starch Granules (With a lambda plate. Magnification x 600)

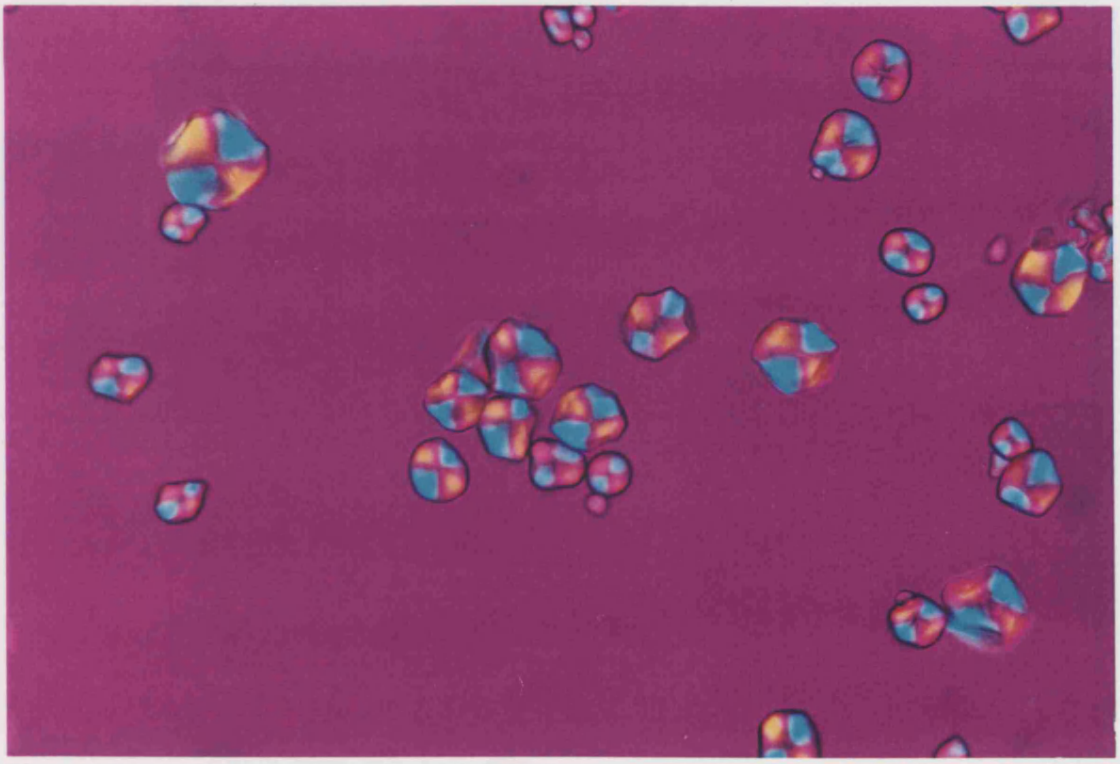


Plate 4.3.13 Polarised Optical Light Micrograph of Maize Starch Granules (With a lambda plate. Magnification x 600). In the photograph, the star-shaped crack in the maize starch granules was probably the result of drying of the starch during its processing.

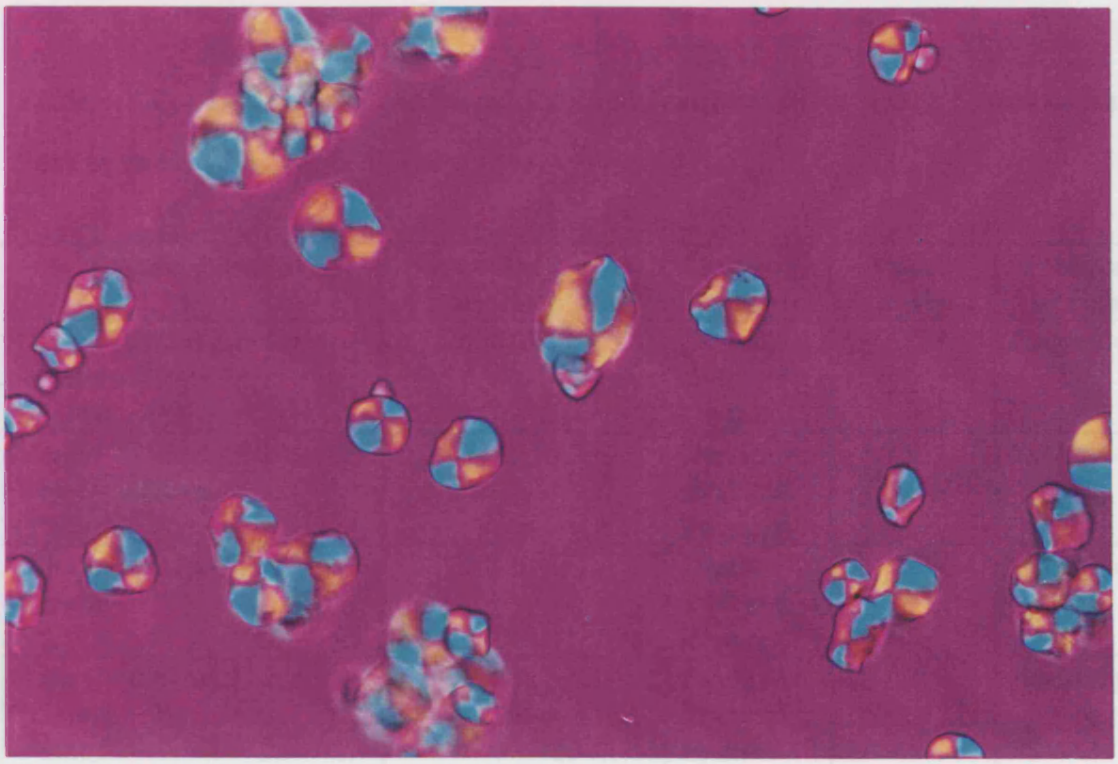


Plate 4.3.14 Polarised Optical Light Micrograph of Waxy Maize Starch Granules

(With a lambda plate. Magnification x 600)

Table 4.3.1 Summary of the Major Physical Features of the Starch Granules As Seen in the Optical Light Micrographs

Starch Sample	Granule Size on Photograph (μm)	Granular Shape	Visible Striations	Position of Hila
Unripe Dessert Banana - Windward Isles (Plate 4.3.6)	11.2 to 52.8	Oyster-shell and irregular	Y	Eccentric
Unripe Dominican Republic Dessert Banana (Plate 4.3.7)	up to 51.2	Oyster-shell and irregular	Y	Eccentric
Unripe Jamaican Dessert Banana (Plate 4.3.8)	20.8 to 51.2	Oyster-shell and irregular	Y	Eccentric
Ripe Colombian Plantain (Plate 4.3.9)	20.8 to 52.8	Elongated or ovoid oyster-shell	Y	Eccentric
Unripe Dominican Republic Cooking Banana (Plate 4.3.10)	24 to 57.6	Elongated oyster-shell	Y	Eccentric
Unripe Ugandan Cooking Banana (Plate 4.3.11)	4.8 to 38.4	Ovoid oyster-shell	Y	Eccentric
Potato (Plate 4.3.12)	12.8 to 56	Round and ovoid oyster-shell	Y	Eccentric
Maize (Plate 4.3.13)	4.8 to 22.4	Irregular, round or polyhedral	N	Central
Waxy Maize (Plate 4.3.14)	6.4 to 27.2	Irregular, round or polyhedral	N	Central

Tables 4.3.2 and 4.3.3 show the data used to construct the particle size distribution profiles of the different starches, and to calculate their particle sizes and their particle size ranges. In Figures 4.3.1 to 4.3.5, all of the starch populations showed unimodal distributions. Two parameters of the distribution were employed in comparing the results, namely the geometric mean particle size (or diameter), M_g, and the geometric standard deviation, σ_g (Table 4.3.4). In addition, the spread of the distribution data was expressed in terms of the range, *i.e.* the difference between the minimum and maximum particle sizes (Table 4.3.4).

The aperture used for measuring the particle size distributions of the *Musa* starch granules (*i.e.* 200 μm) encompassed the range of sizes present in the *Musa* starch samples. Unripe plantain starch and unripe cooking banana starch had particle size ranges of ~7.2 to 72 μm, and unripe dessert banana starch had a particle size range of ~7.2 to 57.5 μm.

The largest geometric standard deviation was shown by potato starch, which had a wide distribution of different granule sizes, which were below and above the range of the particle size limits of the aperture used in the experiment (Figure 4.3.5). Similarly, the smallest starch granules of the cereal starches were also too small to be detected when the 200 μm aperture was used (Figure 4.3.5).

The geometric mean particle sizes of the starches from the different *Musa* types were similar, and ranged from 16.9-22.1 μm. However, with increasing fruit ripeness, the geometric mean particle sizes of the starch granules of the *Musa* fruits decreased. Enzymic degradation was responsible for decreasing the size of the starch granules during fruit ripening, converting the starch granule polysaccharide components into reducing sugars (and also reducing the overall starch content of the fruits).

Rašper (1971) analysed West African plantain starch using the Coulter Counter and the results of M_g and σ_g for cultivar 'Assamiensa' were calculated as 24.64 μm and 1.60 μm, respectively, which were higher values than the M_g (18.9 μm) and σ_g (1.4 μm) reported here for unripe Colombian plantain starch (Table 4.3.4). However, Rašper (1971) also reported higher values of M_g and σ_g for West African maize starch than those of the maize starch in Table 4.3.4.

Table 4.3.2 Particle Sizes used for the Plots of the Particle Size Distributions

Channel Number	Particle Size Range (μm)*	Geometric Mean Particle Size, d_i	% Frequency (W_i) (see Table 4.3.3)
5	6.4 - 8	$d_1 = (6.4 \times 8)^{0.5} = 7.2$	W_1
6	8 - 10	$d_2 = (8 \times 10)^{0.5} = 8.9$	W_2
7	10 - 13	$d_3 = (10 \times 13)^{0.5} = 11.4$	W_3
8	13 - 16	$d_4 = (13 \times 16)^{0.5} = 14.4$	W_4
9	16 - 20	$d_5 = (16 \times 20)^{0.5} = 17.9$	W_5
10	20 - 25	$d_6 = (20 \times 25)^{0.5} = 22.4$	W_6
11	25 - 32	$d_7 = (25 \times 32)^{0.5} = 28.3$	W_7
12	32 - 40	$d_8 = (32 \times 40)^{0.5} = 35.8$	W_8
13	40 - 51	d_9 $(40 \times 51)^{0.5} = 45.2$	W_9
14	51 - 64	d_{10} $(51 \times 64)^{0.5} = 57.1$	W_{10}
15	64 - 80	d_{11} $(64 \times 80)^{0.5} = 71.6$	W_{11}

* Determined from calibrating the instrument with latex particles (the instrument was fitted with a 200 μm aperture).

The Geometric Mean Particle Size, d_i, was used to plot the % Frequency versus particle size, and was also used for the calculation of M_g and σ_g.

Table 4.3.3 % Frequencies (W_is) for the Determination of M_g and σ_g (According to the equations used by Headley and Pfost, 1968)

	% Frequencies (W_is) [Particle Size Ranges (μm)]										
Starch Sample ($n = 2$)	W_1 [6.4-8]	W_2 [8-10]	W_3 [10-13]	W_4 [13-16]	W_5 [16-20]	W_6 [20-25]	W_7 [25-32]	W_8 [32-40]	W_9 [40-51]	W_{10} [51-64]	W_{11} [64-80]
Unripe Dominican Republic Dessert Banana	1.5 ± 0.1	1.7 ± 0.1	4.3 ± 0.4	13.7 ± 0.2	28.0 ± 0.5	27.1 ± 0.6	17.2 ± 0.4	5.8 ± 0.2	1.0 ± 0.2	0.1 ± 0.1	0.0 ± 0.0
Unripe Jamaican Dessert Banana	1.3 ± 0.2	1.7 ± 0.1	5.7 ± 0.8	16.2 ± 2.5	26.8 ± 1.1	25.3 ± 0.8	16.7 ± 2.3	5.6 ± 0.8	0.9 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
Ripe Jamaican Dessert Banana	1.5 ± 0.0	4.2 ± 0.1	14.0 ± 0.5	24.5 ± 0.5	25.9 ± 0.4	17.7 ± 0.4	9.3 ± 0.1	2.7 ± 0.3	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Unripe Colombian Plantain	1.4 ± 0.1	2.1 ± 0.1	7.1 ± 0.3	15.1 ± 0.8	28.7 ± 1.1	29.6 ± 0.8	13.7 ± 0.9	2.5 ± 0.4	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ripe Colombian Plantain	1.4 ± 0.0	2.3 ± 0.0	9.0 ± 0.3	21.7 ± 0.1	32.2 ± 0.1	24.3 ± 0.6	8.0 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Unripe Dominican Republic Cooking Banana	1.5 ± 0.2	1.3 ± 0.0	2.2 ± 0.4	6.4 ± 0.1	21.4 ± 0.4	34.1 ± 0.1	27.4 ± 3.0	7.6 ± 0.1	0.9 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Potato	5.6 ± 0.5	7.2 ± 0.3	11.5 ± 0.9	14.1 ± 0.4	14.8 ± 0.1	14.9 ± 0.5	13.4 ± 0.2	8.9 ± 0.8	6.4 ± 0.1	3.1 ± 0.2	0.6 ± 0.0
Maize	15.0 ± 0.6	18.5 ± 0.4	22.4 ± 0.1	23.9 ± 0.4	16.2 ± 0.6	3.7 ± 0.4	0.4 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Waxy Maize	15.1 ± 0.2	18.5 ± 0.4	23.3 ± 0.6	25.4 ± 0.4	15.4 ± 0.3	2.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

± represents Standard Deviation

Figure 4.3.1 Particle Size Distribution of Unripe Dominican Republic Dessert Banana Starch (day 1)

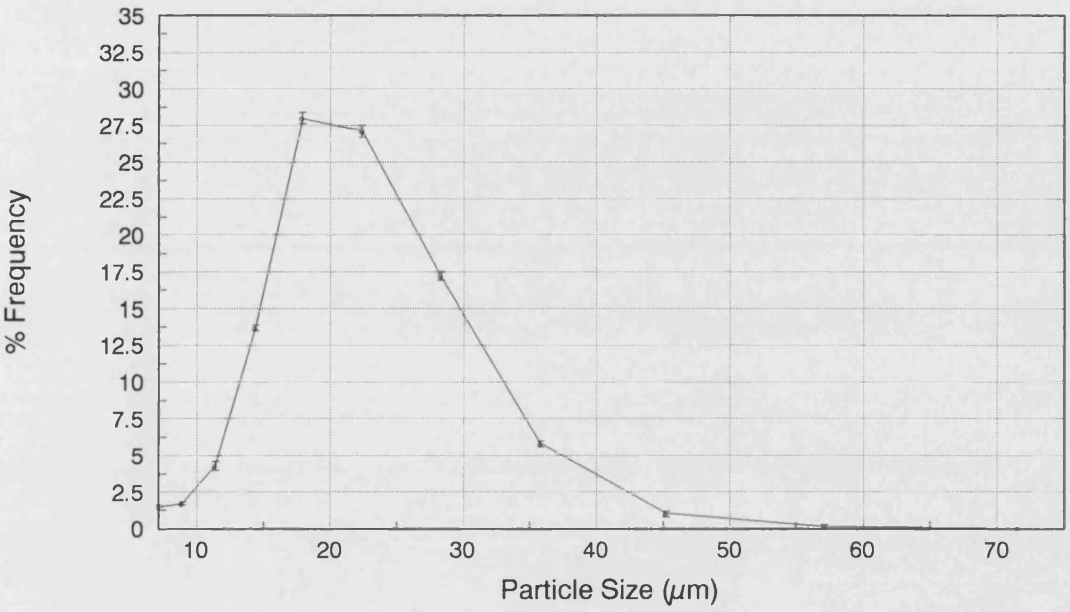
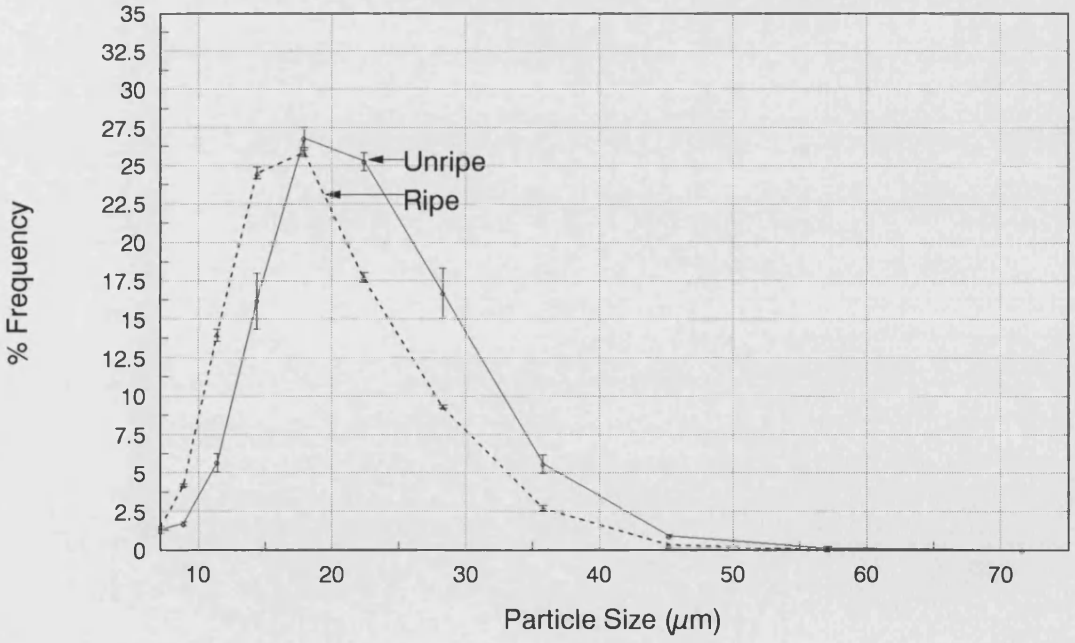


Figure 4.3.2 Particle Size Distributions of Unripe and Ripe Jamaican Dessert Banana Starches (day 1 and day 20, respectively)



Vertical lines indicate Standard Error of the Means

Figure 4.3.3 Particle Size Distributions of Unripe and Ripe Colombian Plantain Starches (day 1 and day 18, respectively)

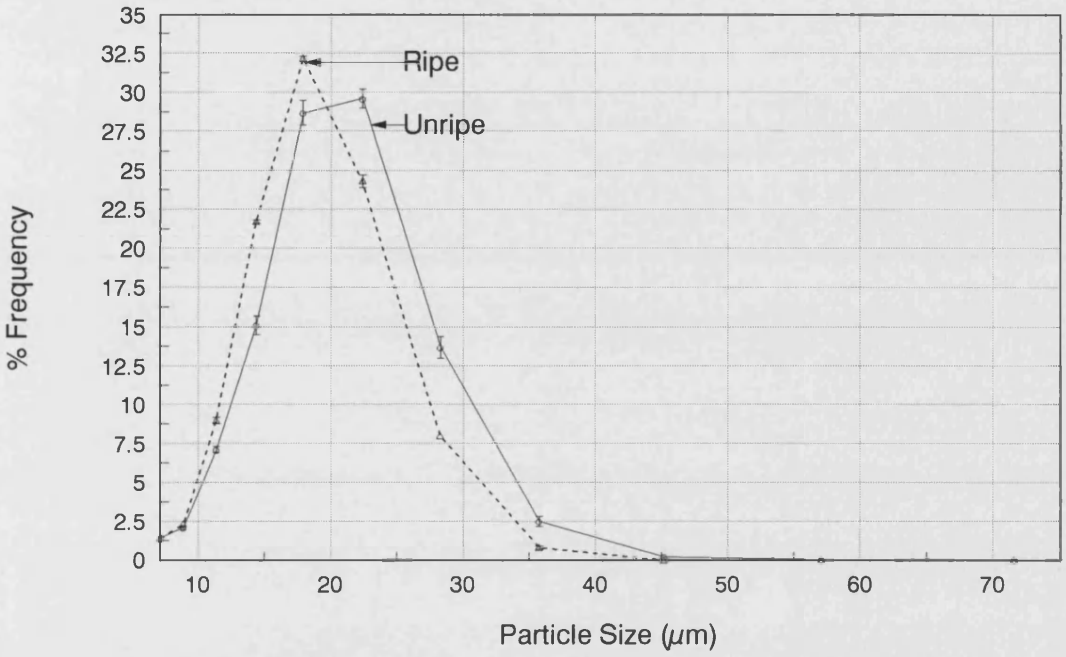
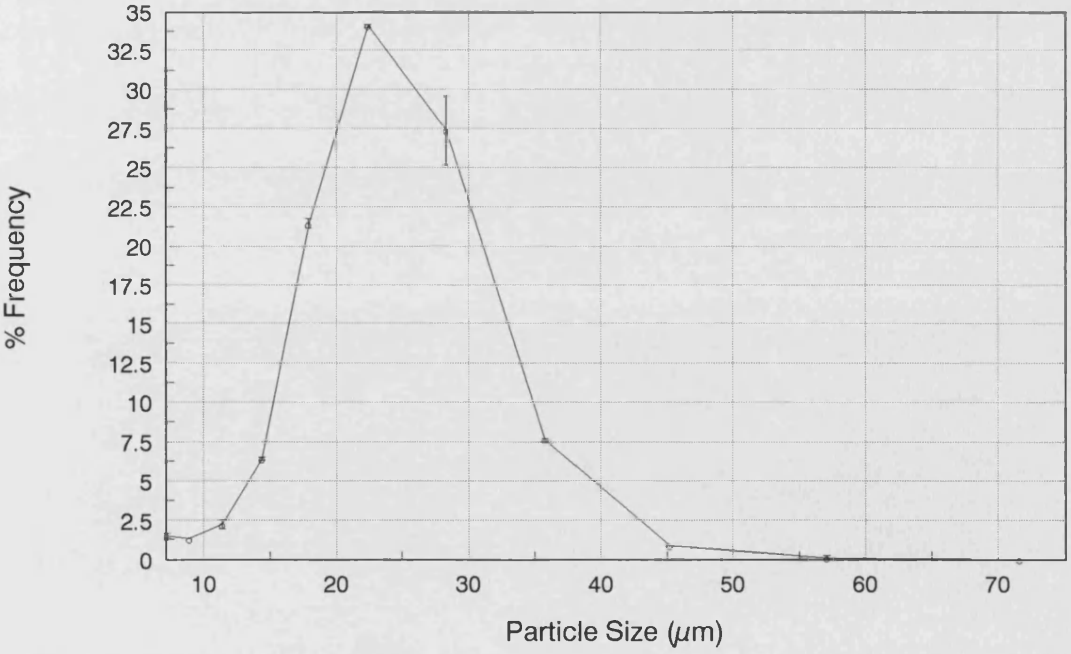
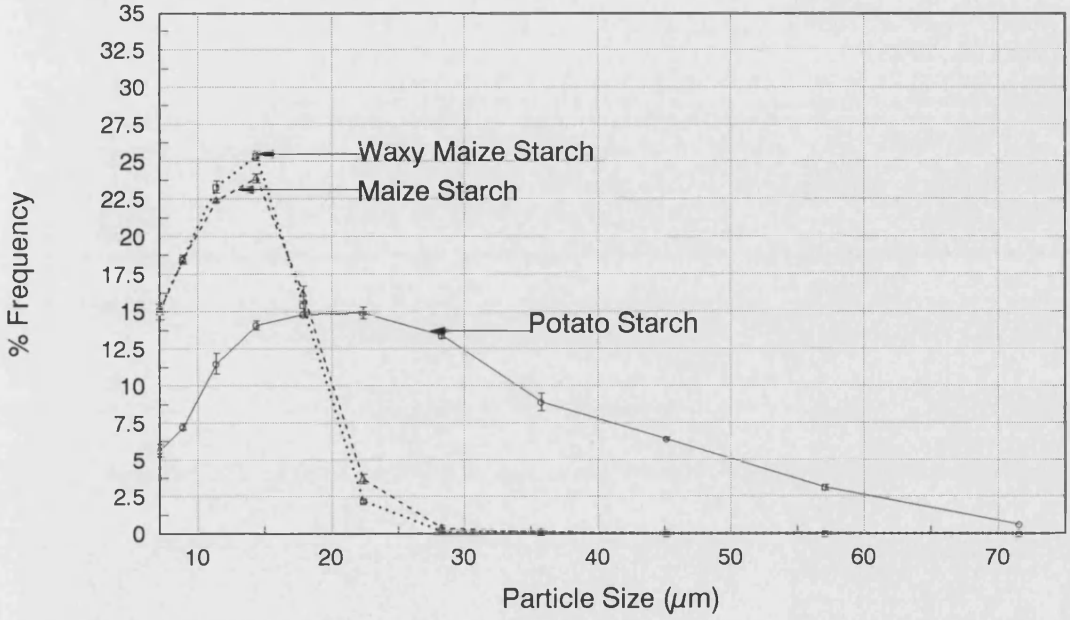


Figure 4.3.4 Particle Size Distribution of Unripe Dominican Republic Cooking Banana Starch (day 3)



Vertical lines indicate Standard Error of the Means

Figure 4.3.5 Particle Size Distributions of Potato Starch, Maize Starch and Waxy Maize Starch



Vertical lines indicate Standard Error of the Means

Table 4.3.4 Coulter Counter Particle Size Distribution Data

Starch Sample	Geometric Mean Particle Size, M_g (μm)	Geometric Standard Deviation, σ_g	Particle Size Range (μm) (from Table 4.3.2)
Unripe Dominican Republic Dessert Banana (day 1) ($n = 2$)	20.1	1.4	7.2 to 57.5
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	19.7	1.4	7.2 to 57.5
Ripe Jamaican Dessert Banana (day 20) ($n = 2$)	16.9	1.4	7.2 to 57.5
Unripe Colombian Plantain (day 1) ($n = 2$)	18.9	1.4	7.2 to 72
Ripe Colombian Plantain (day 18) ($n = 2$)	17.5	1.3	7.2 to 45.5
Unripe Dominican Republic Cooking Banana (day 3) ($n = 2$)	22.1	1.4	7.2 to 72
Potato ($n = 2$)	19.3	1.7	<7.2 to >72
Maize ($n = 2$)	11.9	1.4	<7.2 to 45.5
Waxy Maize ($n = 2$)	11.8	1.4	<7.2 to 36

± represents Standard Deviation

4.4 Conclusions

- The TEM photographs confirmed that the amyloplast membranes around the starch granules in dessert bananas were extremely fragile. The amyloplast membranes were removed from around the starch granules during the blending step of the starch isolation procedure. The physical appearances of the starch granules during the four stages of the starch isolation procedure did not change at all, *i.e.* the starch granules appeared to be unaffected by the starch isolation procedure.

- The SEM photograph revealed that the pulp cells from mature green and unripe *Musa* fruits were packed with starch granules of a variety of different shapes and sizes.

- Under the polarised OLM, the starch granules of the *Musa* starches were generally similar in size and shape, regardless of their *Musa* type or country of origin. The *Musa* starch granules were asymmetrical and oyster-shell in shape, though plantain starch granules and cooking banana starch granules appeared to be slightly more elongated or ovoid than the dessert banana starch granules. The physical characteristics of the potato starch granules as observed under the polarised OLM were similar to those of the *Musa* starch granules, whereas the maize and waxy maize starch granules were very different. This difference was not surprising, as the composition of the cereal starches, especially waxy maize starch (very low AM content) are unlike those of the *Musa* starches. These differences in granular shapes and sizes were probably a result of different structural arrangements of polysaccharides in the starch granules (which is characteristic of a species).

- Using the Coulter Counter technique, the geometric mean particle sizes of the *Musa* starches and potato starch were similar. In contrast, maize starch and waxy maize starch had the smallest geometric mean particle sizes. The geometric mean particle size decreased with increasing ripeness of the fruits from which the starch granules were

isolated, due to the enzymic degradation of the starch polymers during ripening. Thus, the post-harvest age of the fruits is probably a critical factor which will determine the sizes of the starch granules when starch is isolated from the fruits. The particle size ranges of the different *Musa* starches were also similar, irrespective of their *Musa* type, or country of origin. However, potato starch showed a much broader distribution of particle sizes than the *Musa* starches, and the cereal starches had narrower particle size ranges than the *Musa* starches. Therefore, the Coulter Counter demonstrated that there were differences between the geometric mean particle sizes, particle size distributions and their particle size ranges of the starch granules between different plant species (which suggested that genetic factors were responsible for these differences), though significant differences in starch granular sizes were not observed between the different *Musa* types.

Chapter 5

Structural and Molecular Characteristics of *Musa* Starch Granules

5.1 Introduction

According to Banks and Greenwood (1975), the properties of starch granules are due to a combination of their chemical constitution (the ratio of AM to AP) and their physical constitution (involving the organization of these polymers to form a unique structural entity). In this chapter, the structural characteristics of different *Musa* starches were examined using powder X-ray diffraction. The X-ray diffractograms provided information on the percentage of crystalline order, *i.e.* crystallinity, and the crystalline arrangement of the starch polymers within the starch granules. The molecular characteristics of different *Musa* starches were examined by Gel Permeation Chromatography (GPC). The GPC data was used to calculate the ratio of AM to AP. In addition, the GPC data was used to study the molecular characteristics of the major starch granule component (*i.e.* AP). The total AM contents of the *Musa* starches were determined by an iodimetric assay.

5.1.1 Determination of the Structural Characteristics of *Musa* Starch Granules Using Powder X-Ray Diffraction

In starch granules, those double helices which are packed into regular arrays and involved in crystallites provide crystalline order, and they are large or perfect enough to diffract X-rays (see below). Cooke and Gidley (1992) found that granular starches (*e.g.* from wheat, maize, potato, and waxy maize) had less crystalline order than molecular order (or double helix content), and molecular and crystalline order were both disrupted concurrently during gelatinisation. Therefore, Cooke and Gidley (1992) proposed that the forces holding starch granules together are largely at the molecular (double-helical) level, and the enthalpy of gelatinisation primarily reflects the loss of molecular (double-helical) order. Furthermore, crystalline order within starch granules may function only as a means of achieving dense packing, rather than as a primary provider of structural stability (Cooke and Gidley, 1992).

It is the arrangement of the AP component in crystallites which is thought to be responsible for the crystallinity of the native starch granule (French, 1984). These regions of crystallinity (long range order) in native starch granules give rise to characteristic X-ray diffraction patterns. Powder X-ray diffractograms thus provide information about the type of crystal structure of the crystallites of starch granules, and the relative amounts of the crystalline and amorphous phases. The powder X-ray diffraction patterns show relatively broad peaks superimposed on an amorphous 'halo'. The relative intensity of these two features is used to estimate the level of crystalline order (*i.e.* crystallinity) of different native starches.

In order to characterise the *Musa* starches, in an attempt to find structural differences between the different *Musa* types, the crystallinities and types of crystalline pattern of different *Musa* starches were investigated using powder X-ray diffraction (see also section 2.3.5).

5.1.2 Materials and Methods for Powder X-Ray Diffraction

Starches were isolated, according to section 3.2.2, from unripe and ripe Jamaican dessert bananas (from day 1 and day 20, respectively, after purchase of the fruits) and unripe and ripe Colombian plantains (from day 1 and day 18, respectively). As reference starch samples, potato starch and waxy maize starch were also used in the powder X-ray diffraction experiments.

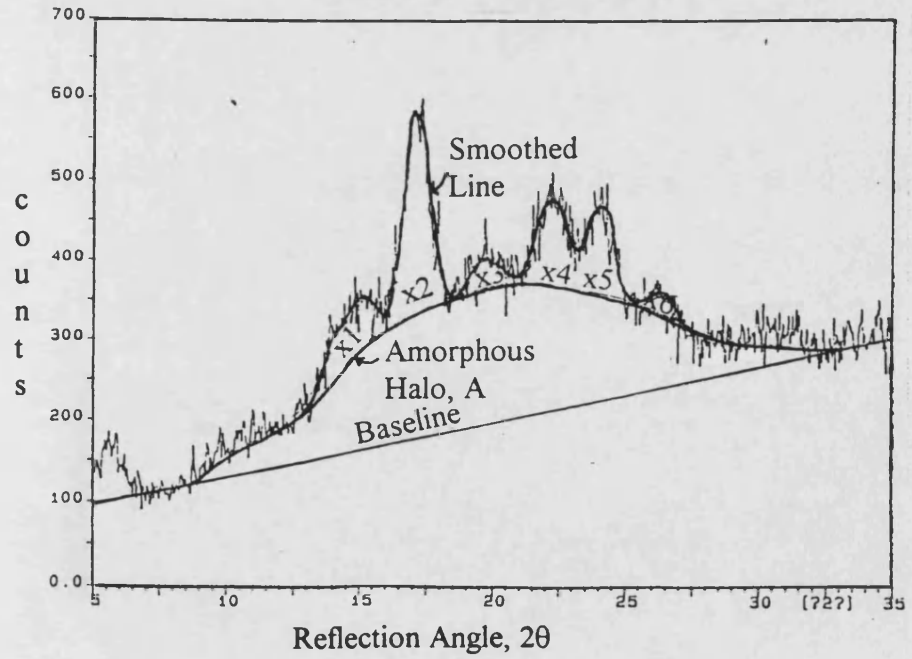
The powder X-ray diffraction patterns were obtained using a Philips powder diffractometer (PCW 1050/1390) mounted on a sealed tube X-ray generator (PCW 1730/10) operating at the Cu-Kα wavelength (1.542 Å). Samples were analysed in a dry and sealed sample-holder unit (to prevent any moisture-induced crystallinity changes).

Powder patterns were analysed quantitatively, by assessing the contribution of the amorphous features to the total diffraction intensity over the angular range 5-30° 2θ (to encompass all of the significant diffraction peaks for starch crystallites). Starch crystallinity was obtained by drawing an estimated baseline and an amorphous halo (from the effect of the non-crystalline material) on the pattern. A smooth line was drawn through the crystalline peak trace (see Figure 5.1.1). Then the trace was cut out and weighed to determine the amounts of amorphous material A, and crystalline material X ($x_1 + x_2 + x_3 + x_4 + x_5 + x_6$). Hence,

$$\% \text{ Crystallinity} = \frac{X}{(X + A)} \times 100$$

The 'integration' procedure was repeated ten times on photocopied traces to reduce human error in the calculation.

Figure 5.1.1 Powder X-Ray Diffractogram of Potato Starch



5.1.3 Results and Discussion

Structural Characteristics of the Starches: Starch Crystallinities and Crystalline Patterns

Zobel (1964) presented a table of the characteristic peaks by which the three types of starch crystalline structures (*i.e.* the A-, B- and C-types) could be identified. This table was used to determine the types of crystalline patterns in Figure 5.1.2. The starch crystallinities and types of crystalline patterns, determined by powder X-ray diffraction, are shown in Table 5.1.1. Waxy maize starch and potato starch were representative of starches with A- and B-type crystalline patterns, respectively. The crystalline structures of the A- and B-type starches differed in the crystalline packing of their double helical molecules and water contents: whereas A-type starch had only 4 water molecules located between the double helices, B-type starch had 36 (see section 2.3.5). In Figure 5.1.2, the diffractograms of the *Musa* starches look similar to the A-type starch, though there appears to be a small amount of B-type character present. This observation, along with the individual diffractogram peaks, suggests that the structural arrangement of the polymers in *Musa* starch granules are C-type (which is intermediate pattern between the A- and B-type starches). This finding is in agreement with Sterling (1968), though it conflicts with some other reports in the literature (*e.g.* Lii *et al.*, 1982; French, 1984).

The crystallinities of the *Musa* starches ranged from 29.5 to 35.6%. The unripe plantain starch and the unripe dessert banana starch had similar crystallinities, though the ripe plantain starch was more crystalline than the ripe dessert banana starch. For both *Musa* types, the crystallinities increased with increasing ripeness of the fruits from which the starch granules were isolated. This increase was greater for plantain than for dessert banana. The higher crystallinity was probably a result of debranching and chain-length shortening due to starch degradative enzymes in the ripe compared to the unripe starch granules. This increase of starch crystallinity with increasing ripeness of the *Musa* fruits,

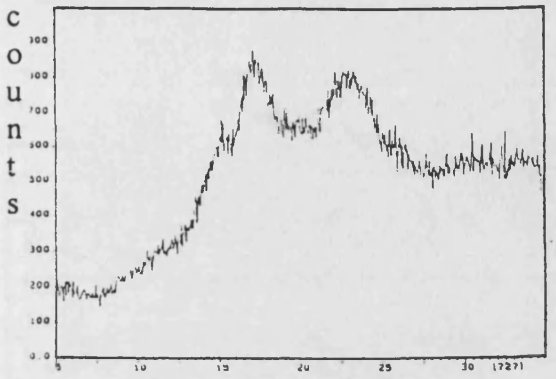
especially for plantain, appears to be analogous to the 'lintnerisation' effect described by Sterling (1960), who demonstrated that during acid treatment of native starch granules ('lintnerization'), the inter-crystalline region was hydrolysed leaving the crystallites essentially intact. The crystallites, no longer separated by amorphous regions, organised laterally and the molecular ends (freed by hydrolytic scission) lined up more readily to increase the relative extent of the crystalline fraction within the treated granule (Sterling, 1960). It is possible that the higher crystallinity of the ripe compared to the unripe *Musa* starches is because the starch degradative enzymes may degrade the amorphous regions of the starch granule more readily than the crystalline regions leading to enhanced crystallinity.

In Figure 5.1.2, the intensities of the diffracted X-rays were noticeably different for the *Musa* starch samples compared to the waxy maize and potato starch samples. This difference was probably due to the packing of the starch sample in the holder.

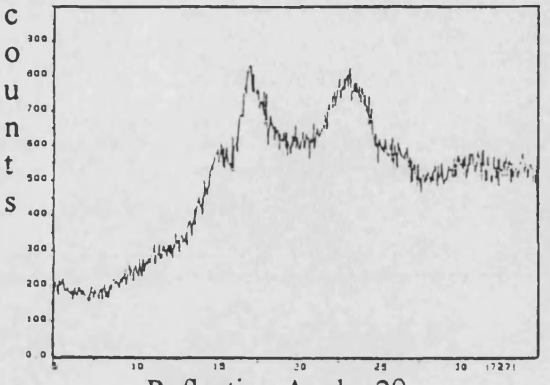
The crystallinity of potato starch is similar to that of the ripe dessert banana starch and the unripe and ripe plantain starches. Though, perhaps more important is the difference between potato starch and the *Musa* starches in the structural arrangements of their starch polymers (B-type and C-type crystalline patterns, respectively), as these starches are from different species. Waxy maize starch (which has a very high content of AP) had a much higher crystallinity than the other starches. This is evidence that the AP component is responsible for the crystallinity of starch. Cooke and Gidley (1992) reported lower crystallinities for both potato starch and waxy maize starch (24% and 28%, respectively) than in Table 5.1.1. Experimental conditions, such as different operator baselines, could be responsible for these differences (Mr. D. Cooke and Dr. M. J. Gidley, pers. comm.).

Figure 5.1.2 Powder X-Ray Diffractograms of *Musa* and Reference Starches

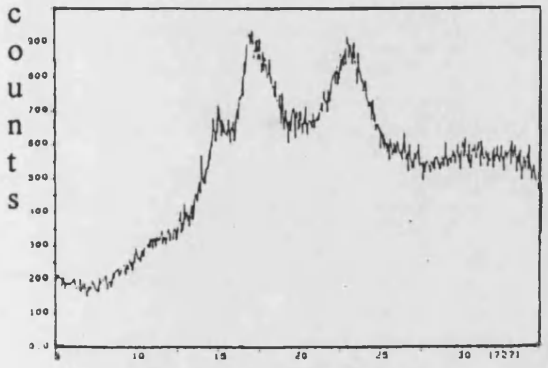
Unripe Jamaican Dessert Banana Starch (day 1)



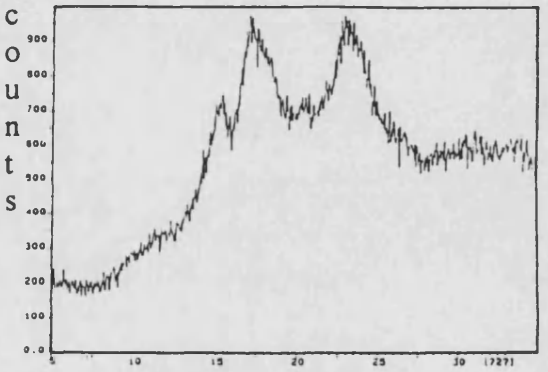
Ripe Jamaican Dessert Banana Starch (day 20)



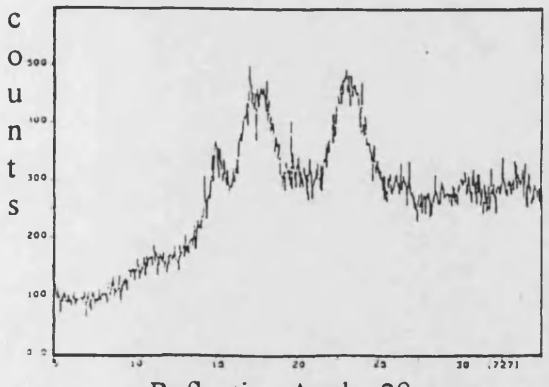
Unripe Colombian Plantain Starch (day 1)



Ripe Colombian Plantain Starch (day 18)



Waxy Maize Starch



Potato Starch

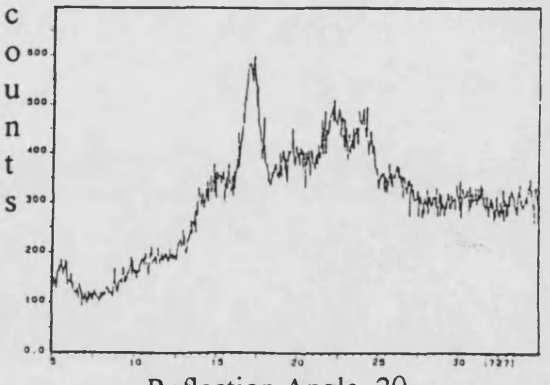


Table 5.1.1 Starch Crystalline Patterns and Crystallinities

Starch Sample	Crystalline Pattern	Crystallinity (%) (Calculated 10 times)
Unripe Jamaican Dessert Banana (day 1)	C	29.5 ± 1.4
Ripe Jamaican Dessert Banana (day 20)	C	31.1 ± 1.3
Unripe Colombian Plantain (day 1)	C	31.6 ± 0.7
Ripe Colombian Plantain (day 18)	C	35.6 ± 0.9
Waxy Maize	A	41.5 ± 1.3
Potato	B	33.2 ± 1.4

± represents Standard Deviation

5.2.1 Determination of the Molecular Characteristics of *Musa* Starches Using GPC

GPC is an analytical procedure for separating small molecules according to their differences in size and shape. The technique of GPC can provide useful information about the polymeric structures of starches. In this chapter, GPC was used to study the content of AM, and the molecular characteristics of the AP component of the starches of different *Musa* types. Cereal and potato starches were also included in the experiments for comparison with the *Musa* starches.

5.2.1.1 Background Theory to GPC

The sample solution is introduced onto the column, which is filled with a rigid-structure (the 'stationary phase') consisting of a gel, or porous organic or inorganic particles, with a defined pore size distribution. The type of column packing material influences the choice of the eluent and the medium for dissolving the starch sample (*e.g.* TSK HW Toyo Soda gels are tolerant to alkaline solutions). The sample resolution in GPC is improved if two columns are used in series (Morrison and Karkalas, 1990). The main role of the solvent or 'mobile phase' is for swelling the gel and keeping the analyte molecules in solution as they are carried through the column (Ravindranath, 1989). The solvent inside the gel (the 'stationary phase') is relatively immobile. The void volume, V_o, of the GPC columns is mainly the interstitial liquid between the packing particles (Yau *et al.*, 1979).

In GPC, molecular size sorting takes place by repeated exchange of the solute molecules between the bulk solvent of the mobile phase and the stagnant liquid phase within the pores of the packing (Yau *et al.*, 1979). Larger solute molecules elute faster than smaller molecules because they have less penetration into the pores of the packing. Molecules which are larger than the pore size of the packing material are completely excluded from the columns.

The raw data of the GPC eluted fractions is a molecular size distribution curve. GPC columns must be calibrated using molecular weight standards of the same polymer structure and under the same experimental conditions as the samples. This is because solute conformation affects the slope and the molecular weight range of the GPC calibration curve (Ravindranath, 1989; Yau *et al.*, 1979). The linear approximation to the calibration curve (a plot of the logarithm of the molecular weights of the standards versus their elution volumes) facilitates molecular weight calculations. With correct column calibration, the raw GPC data of the size-sorted starch molecules can be used to calculate the molecular characteristics of the starch polymers.

In GPC, the AM and AP fractions of the starches emerge as broad peaks, as both fractions are polymolecular and polydisperse. In contrast, Pullulan standards (synthetic α-(1→4)-glucans) used to calibrate GPC columns are of uniform molecular weight (*i.e.* monodisperse), and therefore produce sharper peaks. In this chapter, the AM and AP components of the starches were first completely debranched with *Pseudomonas* isoamylase (EC 3.2.1.68), which is a specific (1→6)-α-D-glucosidase (*i.e.* a debranching enzyme). This produced a population of linear chains which were then fractionated by GPC. From the GPC elution profile data of the starch fractions of the different *Musa* starches, the composition of AM (relative to that of AP), and the molecular characteristics of the AP component, were determined.

5.2.2 Materials and Methods for Gel Permeation Chromatography

Starches were isolated, according to section 3.2.2, from unripe and ripe *Musa* fruits. The fruits comprised unripe Dominican Republic dessert bananas (from day 1 after purchase of the fruits), unripe and ripe Jamaican dessert bananas (from day 1 and day 20, respectively), unripe and ripe Colombian plantains (from day 1 and day 18, respectively), and unripe

Dominican Republic cooking bananas (from day 3). The reference starch samples were maize starch, waxy maize starch, wheat starch and potato starch.

The method was based on that originally developed by Inouchi *et al.* (1983), as modified by Asaoka *et al.* (1984).

Column Set-Up

Two GPC glass columns in series (Wright Scientific Ltd., U.K. and Amicon Ltd., Gloucestershire, U.K., ∅ 1.7 cm x 92 cm, and ∅ 2.2 cm x 100 cm, respectively) were vertically clamped on stands and placed in a temperature-controlled room at 17°C. The packing materials were Fractogel TSK HW-55 (S) and Fractogel TSK HW-50 (S), supplied by Merck-BDH Co., U.K., for the first and second columns, respectively. HW-50 (S) has a pore size of 125 μm, a particle size of 20-40 μm, and a separating range of 500-20,000 for the molecular weight of dextrans. HW-55 (S) has a pore size of 300 μm, a particle size of 20-40 μm, and a separating range of 1,000-200,000 for the molecular weight of dextrans. The packing materials were each washed in distilled water and then soaked overnight in column eluent. The column eluent was 0.2% sodium hydroxide, 0.2% sodium chloride, and 0.02% sodium azide (NaN_3), pH~11. To each of the two packing materials was added triple the volume of column eluent. This was stirred with a glass rod and degassed overnight with an air pump (Charles Austen Pumps Ltd., Surrey, U.K.).

The supernatant was removed and replaced with a freshly degassed volume of eluent three-quarters of the volume of the packing materials. The packing materials were poured down a glass rod into each of the columns to avoid trapping air bubbles. The packed columns were allowed to settle under atmospheric pressure. The columns were connected together and the first column was connected to an LKB Bromma 2132 Microperpex® Peristaltic Pump, running with degassed eluent at a flow rate of approximately

0.33 ml/min. The bottom of the second column was connected to an LKB Bromma 2070 Ultrorac® II fraction collector. Fractions of 2.7 ml were collected in 80 tubes for analysis. The column beds were allowed to equilibrate with a constant flow rate for a few days (replacing freshly degassed eluent daily). Blue Dextran 2000 (Sigma Chem. Co., U.K.) (2 mg/ml) was used to check the homogeneity of the packing and for the presence of any unwanted air bubbles.

Calibration of the Columns

After the columns had been equilibrated and the packing checked with Blue Dextran 2000, the columns were calibrated with Pullulan standards. The same operating conditions as the starch samples were used, but only ~5 mg of each standard was used. The Pullulan standards had molecular weights of 186k, 100k, 48k, 23.7k, 12.2k, and 5.8k (Polymer Laboratories Ltd., Church Stretton, U.K.). The GPC eluted fractions of the Pullulan standards were chemically assayed for their total sugar contents using the phenol-sulfuric acid assay, as described below.

Starch Sample Debranching, GPC Sample Preparation, and Assay of the Eluted Fractions

The starch sample (40 mg) was dissolved in sodium hydroxide (250 µl, 2M) and distilled water (250 µl) in a screw-cap glass bottle, which was capped and placed in a water bath at 40°C for 3 h. The sample was diluted with 3.5 ml distilled water, and approximately 0.9 ml HCl (0.5M), to pH 6.2-6.4 (measured using pH strips). Acetate buffer (5 ml) (0.05 g anhydrous sodium acetate, and 100 ml distilled water, adjusted to pH 3.5 with acetic acid) and isoamylase (0.03 ml) (ex *Pseudomonas amyloderamosa*, EC 3.2.1.68, Hyashibara Biochemical Laboratory Inc., Okayama, Japan, with a molecular weight of ~ 95,000) were added to debranch the starch sample for 24 h at 40°C. The sample was concentrated using a rotary evaporator (Rotavapor, Büchi Laboratories, Switzerland) with

absolute ethanol (Merck-BDH Co., U.K.) (2 x 10 ml), and stored at 5°C. When required for GPC analysis, the concentrated sample was re-diluted with 0.33 ml distilled water and 0.53 ml sodium hydroxide (2M). The sample was centrifuged (Chilspin, MSE, Fisons, U.K.) at 3,300 rpm with 1.24 ml distilled water for 10 min, to remove contaminants. From the supernatant, 1 ml was taken and injected very slowly through the injector assembly of the column system. From the time of injection of the sample, the volume of eluted eluent was measured for about 6.5 h before connecting the column system to the fraction collector. From this point onwards, fractions were collected (80 x 2.7 ml), and each isoamylase-debranched starch sample or molecular weight standard fractionated within 24 h from the time of sample injection.

Analysis of the Eluted Fractions: Determination of the Total Sugar Content Using the Phenol-Sulfuric Acid Assay

The phenol-sulfuric acid assay was used to determine the total sugar contents (carbohydrate concentrations) of each of the eluted fractions of the standards and the starch samples, according to Dubois *et al.* (1956). To 100 µl of each fraction was added 100 µl of 5% (w/v) phenol solution in an Eppendorf tube. Concentrated sulfuric acid (1 ml) was dispensed into each Eppendorf tube, so that the stream of liquid hit the sample directly. After 10 min, the Eppendorf tubes were whirlimixed and left to stand at room temperature for 30 min. The solution was mixed again before reading the absorbance of the solutions at 490 nm (Philips PU 8620 UV/VIS/NIR spectrophotometer). The carbohydrate concentration of each eluted fraction was calculated from a calibration curve obtained by plotting the absorbance versus the concentration of glucose, using concentrations ranging from 15-300 µg/ml of D-glucose (Diagnostic Glucose Standard solution, Sigma Chem. Co., U.K.). Distilled water was used as a blank for the samples and the molecular weight standards.

The molecular weights of the AP fractions of the starches were derived from the linear calibration curve, constructed from the plot of the logarithm of the molecular weights of the Pullulan standards versus their elution volumes.

Identification of Each GPC Eluted Fraction

It is a characteristic of AM and AP polymers to react differently with iodine in solution. Therefore, when starch has been fractionated into its constituent AM and AP polymers using GPC, the AM and AP fractions can be distinguished from each other by their wavelengths of maximum absorbance (λ_{max}) and the characteristic colour of their AM- or AP-iodine complex. Thus, in order to define the starch molecular component of each fraction of the GPC elution profiles (constructed from the phenol-sulfuric acid assay data), each eluted fraction was neutralised with 0.2M acetic acid and 0.1M NaOH to within $\pm$ 0.1 pH units (as iodine staining for λ_{max} is sensitive to pH). An aliquot of neutralised sample (collected fraction) was mixed with an aliquot of I_2-KI solution (0.2%, w/v and 2%, w/v, respectively) and mixed. The colour was a function of the chain length of the linear regions of the constituent polymers. Each fraction was scanned for its wavelength of maximum absorbance (λ_{max}) between 450-740 nm (Philips PU 8740 UV/VIS Scanning Spectrophotometer). Distilled water was used as a blank. In this way, the colour of the starch-iodine fractions and their wavelengths of maximum absorbance (λ_{max}) identified the fractions on the GPC elution profiles as either AM or AP.

Data Analysis

GPC elution profiles were constructed from the phenol-sulfuric acid assay data (Figure 5.2.2). From the linear calibration curve constructed from the Pullulan standards (Figure 5.2.1), the molecular weights (MWs) of the eluted AP fractions were calculated. From the latter data, the weight-average molecular weight (M_w), the degree of polymerisation (DP), and the weight-average degree of polymerisation (DP_w) of the AP

fractions were determined. For the calculation of DP, a glucose unit was defined as glucose anhydride of molecular weight 162, and the DP of the linear fractions of the debranched starches was calculated as MW (at peak) divided by 162. For the purpose of AM and AP quantitation in the starches, the GPC elution profiles were cut at a point (denoted by an arrow, Figure 5.2.2), which divided the AM fraction (Fr. I) from the other fractions. The AM content was calculated as the area corresponding to the AM fraction in relation to the total GPC elution profile area. In addition, the percentages of the other eluted fractions, and the ratio of the two (main) AP fractions (Fr. III: Fr. II) were calculated.

5.2.3 Results and Discussion

Molecular Characteristics of the Starches

Column Calibration

The plot of the logarithms of the molecular weights of the Pullulan standards versus their elution volumes was linear in the lower molecular weight region (r = 0.999, Figure 5.2.1). In GPC, it is common for higher molecular weight fractions to elute near the void volume of the system, and this was the reason why the 186k and 100k molecular weight Pullulan standards deviated from the straight line of linear regression. Therefore, the molecular weights (MWs) of the eluted starch fractions (Table 5.2.3) were determined using the linear regression of the lower molecular weight Pullulan standards (Figure 5.2.1). The weight-average molecular weight (M_w) (Table 5.2.4), the degree of polymerisation (DP) (Table 5.2.5), and the weight-average degree of polymerisation (DP_w) (Table 5.2.6) of the eluted starch fractions were calculated from the molecular weight data (Table 5.2.3).

λ_{max} of the Eluted Fractions

The fractions of the GPC elution starch profiles, *i.e.* the AM and AP polymers, were distinguished by the maximum wavelengths (λ_{max}) of their iodine-carbohydrate complex curves (Table 5.2.1). The λ_{max} determination, using I_2-KI solution, confirmed that the first peak of each of the GPC elution profiles (Fr. I) was pure AM, as its maximum absorbance was between 620 and 630 nm (the AM-I_2-KI complex was an intense blue colour). The intermediate fraction, Fr. Int., gave a deep-blue colour with iodine, similar to that of the AM-iodine colour but the depth of the colour was less. The maximum absorbance was 588-600 nm, which was between the maximum absorbances of AP (540 nm) and AM (620 nm). At its maximum absorbance, the colour of the AP-I_2-KI complex was brown.

Descriptions of the Fractions

Figure 5.2.2 shows the GPC elution profiles of the isoamylase-debranched *Musa*, cereal and potato starches. Each profile shows the AM fraction (Fr. I), the intermediate fraction (Fr. Int.), followed by the bimodal distribution of the AP component (Fr. II and Fr. III), except for maize starch and wheat starch which had trimodal AP distributions. (Though not clear in the 'smoothed' gel permeation chromatogram shown in Figure 5.2.2, the trimodal distribution of maize starch was clearly observed in the 'raw data' gel permeation chromatograms.) Fr. Int. was probably composed of large molecules derived from the debranched AM fraction, which were intermediate in molecular weight between the AM and AP fractions (see section 2.3.3.1). Fr. II was mainly composed of longer chain AP, from the B-chains, and possibly very long A-chains (see the cluster model of AP, Figure 2.3.2). Fr. III was mainly composed of the long A-chains, and possibly shorter B-chains, of the AP component. The third AP fraction, seen only in the GPC elution profiles of maize starch and wheat starch (Fr. IV, Figure 5.2.2 and Table 5.2.2), was probably composed of much shorter (outer) A-chains of AP.

In Figure 5.2.2, the AP fractions on the GPC elution profiles of the *Musa* starches were similar to those of the cereal starches and potato starch. This was suggestive of similarities in the molecular characteristics of the starches of the different plant species. Kayisu and Hood (1981) also suggested that the structure of banana AP was similar to that of potato and cereal APs.

Relative Proportions of the Eluted Fractions

Table 5.2.2 shows the percentages of the fractions eluted by GPC for the *Musa* and reference starches. Maize starch had the highest and waxy maize starch the lowest AM contents (Fr. I). The AM contents of wheat starch and potato starch were similar to those of the cooking banana and plantain starches. The AM contents of the *Musa* starches

ranged from 16.8-23.9%. The plantain starches had similar AM contents to the cooking banana starch, and the dessert banana starches had lower AM contents. Kayisu and Hood (1981) reported that the AM content of starch which had been isolated from green banana flour was 16%. This value was similar to that of the dessert banana starches (*i.e.* Fr. I) in Table 5.2.2.

All of the starches had similar proportions of Fr. Int., except for that of waxy maize starch, which was significantly less. The starches also had similar proportions of Fr. II (longer chain AP, or B-chains), except for maize starch and wheat starch which had the least amount of Fr. II. Waxy maize starch had the highest proportion of Fr. III (shorter chain AP, or A-chains) than the other starches. The starches of the different unripe and ripe *Musa* types and potato starch had similar proportions of Fr. III. Maize starch and wheat starch had the lowest proportions of shorter chain AP, though both of these starches also contained another even shorter chain fraction, *i.e.* Fr. IV.

Ratio of Fr. III to Fr. II

The ratio of the total carbohydrate content of Fr. III to that of Fr. II (*i.e.* Fr. III: Fr. II) may be used as an index of the extent of branching of AP (*i.e.* the higher the ratio, the higher the degree of branching) (Inouchi *et al.*, 1983; Inouchi *et al.*, 1987). In Table 5.2.2, the ratio of Fr. III to Fr. II was determined on a weight basis.

The cereal starches, especially waxy maize starch, had the highest proportions of Fr. III: Fr. II (A-chains: B-chains). Therefore, the cereal starches had more branching of their AP polymers compared to the *Musa* starches. Potato starch had the lowest Fr. III to Fr. II ratio, *i.e.* it had the least branched AP component, compared to the other starches. The *Musa* starches had similar Fr. III to Fr. II ratios which ranged from 1.5: 1 to 1.6: 1. However, Kayisu and Hood (1981) reported a much higher AP chain ratio (Fr. III: Fr. II)

of starch which had been isolated from green banana flour, also determined on a weight basis, of 2.1: 1.

Molecular Weight (MW): Measured at the Peak of Each AP Fraction

The molecular weights, measured at the peak of each fraction, are shown in Table 5.2.3. AM is a very large heterogeneous polymer which elutes near the void volume of the GPC column system (section 5.2.1.1). Because of this, using the GPC technique, it was not accurate to attempt any calculations for the AM fraction, other than its proportion in the starch granule relative to that of the AP component. The MWs of Fr. II (B-chains) and Fr. III (A-chains) of all of the starches were similar, and there did not appear to be any obvious species specific trends.

Weight-Average Molecular Weight (M_w) of Each AP Fraction

As well as the peak MW, the weight-average molecular weight was also calculated. The weight-average molecular weights (M_ws) of the AP fractions were determined because each fraction on the GPC elution profile (Figure 5.2.2) was composed of many different molecular sizes. Thus, the MW is probably better expressed on a weight-average basis, *i.e.* M_w, as this can give an indication of the lengths of the chains of which the overall AP molecule is composed. In Table 5.2.4, the M_ws of Frs. II and III ranged from 8-10k and 2-4k, respectively. The M_ws of Frs. II and III of the starches were generally similar, though wheat starch had the lowest M_w of Fr. II (B-chains), and maize starch and wheat starch displayed higher M_ws in Fr. III than the other starches.

Degree of Polymerisation (DP): Measured at the Peak of Each AP Fraction

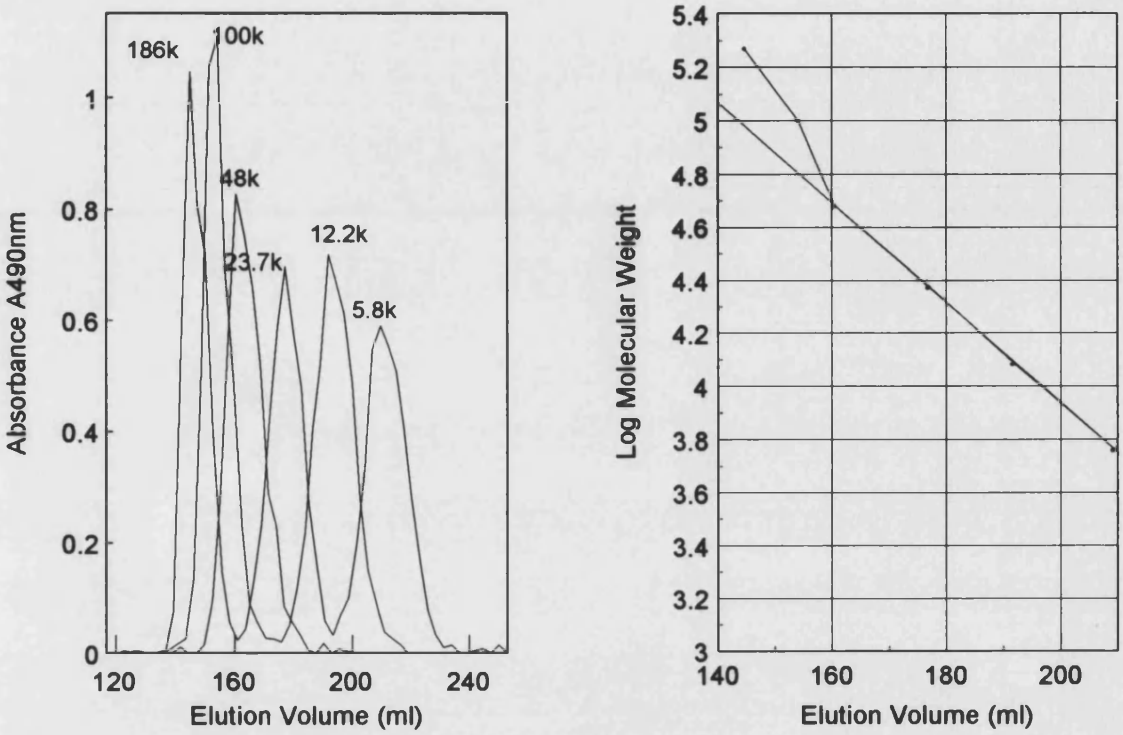
The DPs of the AP fractions are shown in Table 5.2.5. The DP of Frs. II and III of the *Musa* starches ranged from 42-46 and 12-14, respectively. These values were similar to the values obtained by Kayisu and Hood (1981), who reported that starch which had been

isolated from green banana flour had AP populations of DP 45 and DP 15. However, Kayisu and Hood (1981) determined the DP from chemical assay, *i.e.* total glucose units/total reducing groups, and did not use the molecular weight of the fraction divided by the molecular weight of glucose anhydride as was used in the experiment described here.

Weight-Average Degree of Polymerisation (DP_w) of Each AP Fraction

The DP was expressed on a weight basis, *i.e.* DP_w, in Table 5.2.6, to give a better indication of the lengths of chains of which the overall AP molecule is composed. The DP_w of Frs. II and III of the three different *Musa* cultivars ranged from 53-60 and 13-14, respectively.

Figure 5.2.1 GPC Elution Profiles of the Pullulan Standards (Combined Profiles) and Plot of the Logarithms of the Molecular Weights of the Pullulan Standards versus their Elution Volumes

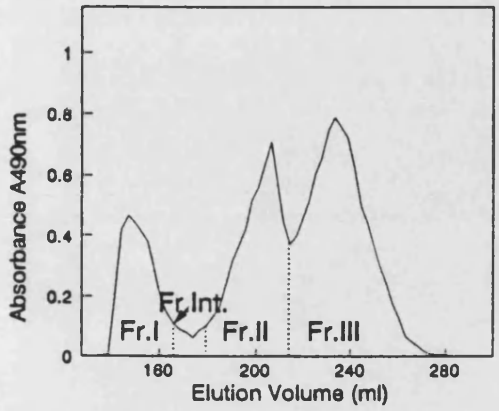


The Pullulan standards comprised those with molecular weights of 186k, 100k, 48k, 23.7k, 12.2k, and 5.8k. The lower molecular weight Pullulan standards were used to derive a linear regression.

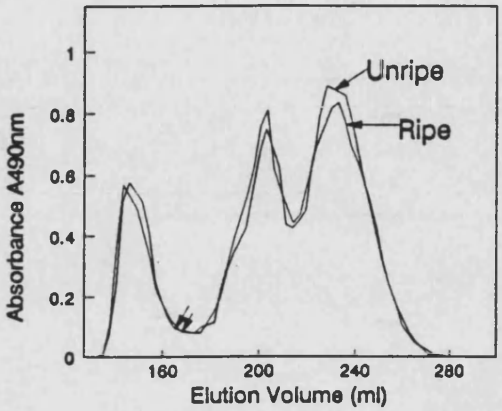
Regression coefficient, $r = 0.999$, Number of points = 4, b = -0.019, and a = 7.700, where, $y = a + bx$

Figure 5.2.2 GPC Elution Profiles of the Isoamylase-Debranched Starches

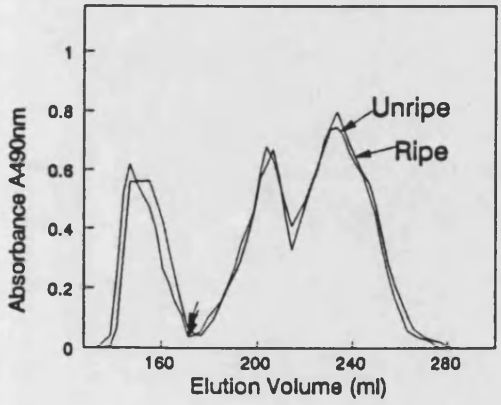
Unripe Dominican Republic Dessert Banana Starch



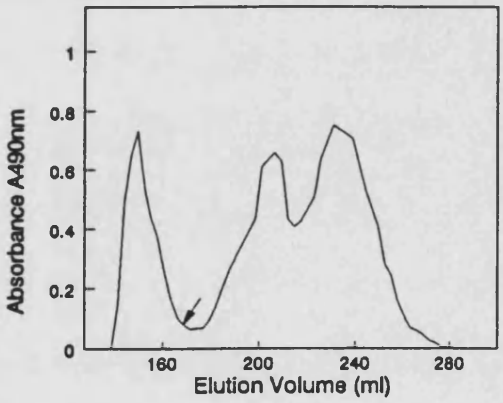
Unripe and Ripe Jamaican Dessert Banana Starches



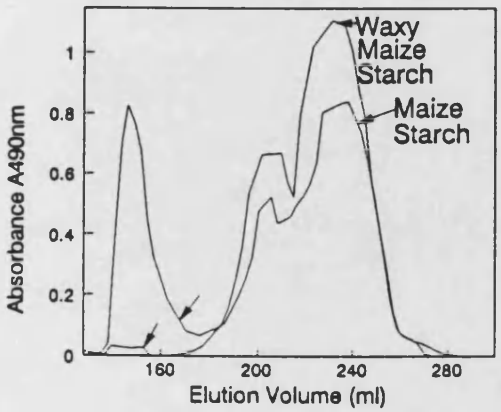
Unripe and Ripe Colombian Plantain Starches



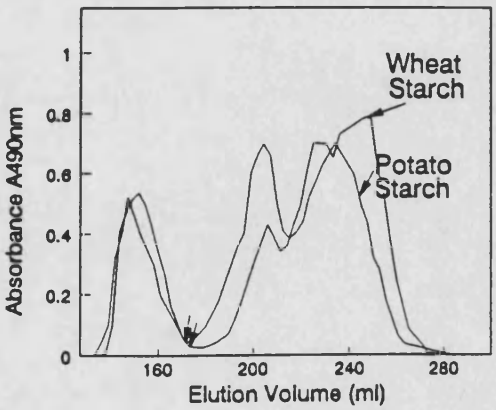
Unripe Dominican Republic Cooking Banana Starch



Maize Starch and Waxy Maize Starch

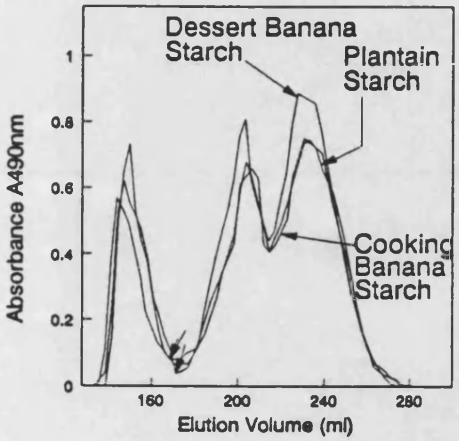


Wheat Starch and Potato Starch

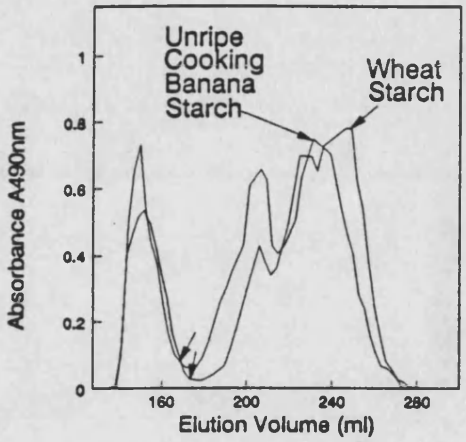


The same gel permeation chromatograms are shown below as combined GPC profiles for ease of comparison between the different starches.

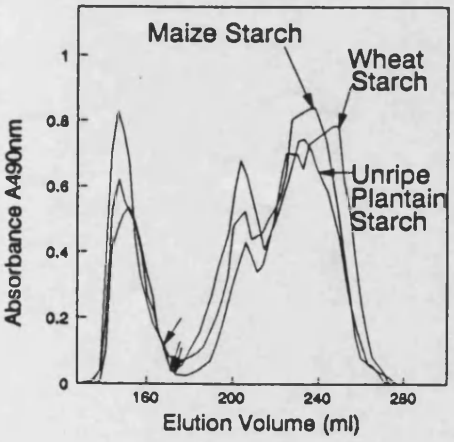
Starches of Different Unripe Musa Types



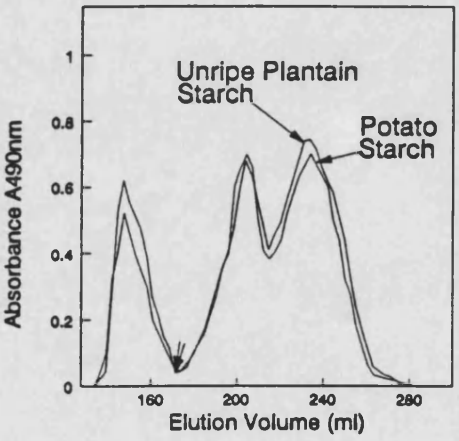
Unripe Dominican Republic Cooking Banana Starch and Wheat Starch



Maize Starch, Wheat Starch and Unripe Colombian Plantain Starch



Potato Starch and Unripe Colombian Plantain Starch



↓ denotes point where GPC profile was divided for the calculation of the ratio of AM to AP, i.e. elution limit of the AM component

Table 5.2.1 λ_{max} of the Eluted Fractions

Division of the eluted fractions on the GPC profiles, based on their wavelengths of maximum absorbance (λ_{max}) from the starch-I_2 tests.

GPC Eluted Fraction	Wavelength of Maximum Absorbance, λ_{max}
Fr. I	$\lambda_{max} \geq 620$ nm (characteristic for AM)
Fr. Int.	620 nm$> \lambda_{max} \geq 588$ nm
Fr. II	588 nm$> \lambda_{max} > 525$ nm
Fr. III	$\lambda_{max} \leq 525$ nm

Table 5.2.2 Relative Proportions of AM and AP in the Starch Samples (determined from the carbohydrate contents of the eluted fractions)

Starch Sample	% Fr. I (AM)	% Fr. Int.	% Fr. II (AP)	% Fr. III (AP)	% Fr. IV (AP)	Fr. III: Fr. II
Unripe Dominican Republic Dessert Banana (day 1) ($n = 3$)	16.8 ± 1.0	1.5 ± 0.3	31.5 ± 1.9	50.3 ± 0.8	-	1.6: 1
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	17.8 ± 0.4	1.7 ± 0.4	31.4 ± 0.1	49.1 ± 0.1	-	1.6: 1
Ripe Jamaican Dessert Banana (day 20) ($n = 3$)	17.9 ± 0.4	1.6 ± 0.3	31.3 ± 2.4	49.2 ± 2.5	-	1.6: 1
Unripe Colombian Plantain (day 1) ($n = 4$)	23.0 ± 1.1	0.8 ± 0.5	30.1 ± 0.9	46.0 ± 1.9	-	1.5: 1
Ripe Colombian Plantain (day 18) ($n = 3$)	23.9 ± 2.4	0.7 ± 0.3	29.0 ± 3.3	46.4 ± 2.4	-	1.6: 1
Unripe Dominican Republic Cooking Banana (day 3) ($n = 3$)	21.6 ± 0.6	0.9 ± 0.2	29.4 ± 1.1	48.1 ± 1.0	-	1.6: 1
Maize ($n = 2$)	25.4 ± 0.1	1.7 ± 0.5	15.1 ± 1.1	29.3 ± 1.0	28.5 ± 0.7	1.9: 1
Waxy Maize ($n = 2$)	1.2 ± 0.8	0.2 ± 0.0	29.8 ± 1.9	69.0 ± 2.8	-	2.3: 1
Wheat ($n = 2$)	23.0 ± 1.0	0.9 ± 0.6	14.9 ± 2.2	26.7 ± 1.4	34.6 ± 3.9	1.8: 1
Potato ($n = 3$)	21.2 ± 0.9	0.5 ± 0.2	34.6 ± 1.1	43.8 ± 1.3	-	1.3: 1

± represents Standard Deviation

Table 5.2.3 Molecular Weight (MW) of Each AP Fraction

Starch Sample	MW of Fr. II (AP)	MW of Fr. III (AP)	MW of Fr. IV (AP)
Unripe Dominican Republic Dessert Banana (day 1) ($n = 3$)	6.7 ± 0.3k	2.0 ± 0.0k	-
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	6.9 ± 0.9k	2.3 ± 0.5k	-
Ripe Jamaican Dessert Banana (day 20) ($n = 3$)	7.4 ± 0.7k	2.0 ± 0.3k	-
Unripe Colombian Plantain (day 1) ($n = 4$)	7.1 ± 0.8k	2.0 ± 0.5k	-
Ripe Colombian Plantain (day 18) ($n = 3$)	7.1 ± 0.6k	2.3 ± 0.4k	-
Unripe Dominican Republic Cooking Banana (day 3) ($n = 3$)	7.0 ± 1.2k	1.9 ± 0.3k	-
Maize ($n = 2$)	6.8 ± 0.0k	2.7 ± 0.0k	1.7 ± 0.0k
Waxy Maize ($n = 2$)	6.8 ± 1.6k	1.9 ± 0.5k	-
Wheat ($n = 2$)	6.6 ± 0.2k	2.3 ± 0.5k	1.1 ± 0.1k
Potato ($n = 3$)	7.3 ± 0.9k	2.3 ± 0.3k	-

± represents Standard Deviation

Table 5.2.4 Weight-Average Molecular Weight (M_w) of Each AP Fraction

$$M_w = \frac{\Sigma(C_i \cdot MW_i)}{\Sigma C_i}$$

where,

M_w = Weight-average molecular weight

C_i = Carbohydrate concentration of each fraction

MW_i = Molecular Weight of each fraction

Starch Sample	M_W of Fr. II	M_W of Fr. III	M_W of Fr. IV
Unripe Dominican Republic Dessert Banana (day 1) ($n = 3$)	9.7 ± 0.4k	2.2 ± 0.1k	-
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	8.7 ± 0.6k	2.1 ± 0.2k	-
Ripe Jamaican Dessert Banana (day 20) ($n = 3$)	9.1 ± 0.5k	2.1 ± 0.2k	-
Unripe Colombian Plantain (day 1) ($n = 4$)	8.5 ± 0.8k	2.1 ± 0.2k	-
Ripe Colombian Plantain (day 18) ($n = 3$)	9.3 ± 0.3k	2.2 ± 0.2k	-
Unripe Dominican Republic Cooking Banana (day 3) ($n = 3$)	9.2 ± 0.1k	2.2 ± 0.1k	-
Maize ($n = 2$)	9.1 ± 0.0k	3.5 ± 0.0k	1.5 ± 0.0k
Waxy Maize ($n = 2$)	8.1 ± 0.5k	2.1 ± 0.1k	-
Wheat ($n = 2$)	7.7 ± 0.2k	3.0 ± 0.3k	1.2 ± 0.1k
Potato ($n = 2$)	9.0 ± 0.6k	2.1 ± 0.2k	-

± represents Standard Deviation

Table 5.2.5 Degree of Polymerisation (DP) of Each AP Fraction

$$DP = \frac{MW\ (at\ peak)}{162}$$

Starch Sample	DP of Fr. II	DP of Fr. III	DP of Fr. IV
Unripe Dominican Republic Dessert Banana (day 1) ($n = 3$)	41.6 ± 2.0	12.4 ± 0.3	-
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	42.8 ± 5.4	14.2 ± 2.9	-
Ripe Jamaican Dessert Banana (day 20) ($n = 3$)	45.7 ± 4.5	12.2 ± 1.9	-
Unripe Colombian Plantain (day 1) ($n = 4$)	43.6 ± 4.6	12.5 ± 3.2	-
Ripe Colombian Plantain (day 18) ($n = 3$)	44.0 ± 3.7	14.3 ± 2.2	-
Unripe Dominican Republic Cooking Banana (day 3) ($n = 3$)	43.2 ± 7.4	11.9 ± 2.0	-
Maize ($n = 2$)	42.0 ± 0.0	16.5 ± 0.0	10.3 ± 0.0
Waxy Maize ($n = 2$)	42.2 ± 10.0	11.7 ± 3.0	-
Wheat ($n = 2$)	40.9 ± 1.5	16.2 ± 3.3	6.7 ± 0.8
Potato ($n = 3$)	44.8 ± 5.6	14.5 ± 1.7	-

± represents Standard Deviation

Table 5.2.6 Weight-Average Degree of Polymerisation (DP_w) of Each AP Fraction:

$$DP_w = \frac{M_w}{162}$$

Starch Sample	DP_w of Fr. II	DP_w of Fr. III	DP_w of Fr. IV
Unripe Dominican Republic Dessert Banana (day 1) ($n = 3$)	59.6 ± 2.6	13.9 ± 0.5	-
Unripe Jamaican Dessert Banana (day 1) ($n = 2$)	53.5 ± 3.7	12.8 ± 1.5	-
Ripe Jamaican Dessert Banana (day 20) ($n = 3$)	55.9 ± 3.3	12.8 ± 1.0	-
Unripe Colombian Plantain (day 1) ($n = 4$)	52.7 ± 4.7	12.7 ± 1.1	-
Ripe Colombian Plantain (day 18) ($n = 3$)	57.3 ± 1.9	13.6 ± 1.2	-
Unripe Dominican Republic Cooking Banana (day 3) ($n = 3$)	56.6 ± 0.7	13.4 ± 0.4	-
Maize ($n = 2$)	55.9 ± 0.0	21.6 ± 0.1	9.0 ± 0.1
Waxy Maize ($n = 2$)	49.8 ± 2.9	13.2 ± 0.9	-
Wheat ($n = 2$)	47.6 ± 0.9	18.8 ± 2.1	7.5 ± 0.8
Potato ($n = 3$)	55.9 ± 3.5	13.1 ± 1.0	-

± represents Standard Deviation

Relationship Between Chain Length and Crystalline Pattern

In the GPC and powder X-ray diffraction experiments, a relationship can be seen between the type of crystallinity pattern and the ratio of Fr. III to Fr. II (A-chains: B-chains). Waxy maize starch granules have an A-type crystalline pattern (Table 5.1.1), and waxy maize starch had the highest Fr. III to Fr. II ratio (Table 5.2.2); potato starch granules have a B-type crystalline pattern (Table 5.1.1), and potato starch had the lowest Fr. III to Fr. II ratio (Table 5.2.2); the *Musa* starch granules were found to have C-type crystalline patterns (Table 5.1.1), and the *Musa* starches had Fr. III to Fr. II ratios which were in between those of waxy maize starch and potato starch (Table 5.2.2). These findings correlate with those of Hizukuri (1985), who reported that the AP molecules of A-type starches had larger amounts of the short-chain fractions (*i.e.* Fr. III), than those of the B-type starches. In addition, Hizukuri (1985) reported that the observed crystal type of AP depended on weight-average chain length, *i.e.* short (26), long (36), and intermediate (28) chains gave A, B, and C patterns, respectively. However, from the results of the experiments in this chapter, this relationship between the DPs and DP_ws of the starches and their type of crystallinity pattern is unclear (Tables 5.2.5 and 5.2.6).

5.3.1 Determination of the Total Amylose Contents of Starches Using the Iodimetric Assay

In this chapter, the iodimetric assay was used to determine the total AM contents of *Musa* starches. The iodimetric method, as described by Morrison and Laignelet (1983), is a widely used method for the determination of the total AM content of cereal starches. The method relies on the binding of the AM component with iodine (see also section 2.3.3.3), which gives a measurement described as the Blue Value. The assay involves the dissolution of the starch in DMSO and urea at 100°C. The starch polymers are then precipitated with ethanol to free them of any complexing lipids which would compete with the iodine. Next, the precipitate is redissolved in DMSO/urea and mixed with an I_2/KI

solution. The absorbance of the solutions is read at 635 nm. In the experiment described in this chapter, the Blue Values of the native starches are defined as the absorbance at A635 nm per 10 mg dry starch in 100 ml at 20°C. These values are corrected for temperature to 20°C. The total AM contents of the *Musa* starches and cereal starches were calculated using a regression equation devised by Morrison and Laignelet (1983). This equation relates the Blue Values of cereal starches to their AM contents which have been determined by GPC.

5.3.2 Materials and Methods for the Iodimetric Assay

Starches were isolated, according to section 3.2.2, from both unripe and ripe *Musa* fruits. The fruits comprised unripe Dominican Republic dessert bananas (from day 1 after purchase of the fruits), unripe and ripe Jamaican dessert bananas (from day 1 and day 20, respectively), unripe Dominican Republic plantains (from day 2), unripe and ripe Colombian plantains (from day 1 and day 18, respectively), and unripe Dominican Republic cooking bananas (from day 3). The reference starch samples comprised maize starch, waxy maize starch, and the high AM starches, Hylon V starch and Hylon VII starch.

The moisture contents of all of the starches were determined prior to the iodimetric assay, according to section 3.2.4.

Analytical grade dimethylsulfoxide (DMSO), urea, iodine (I_2) and potassium iodide (KI) were supplied by Sigma Chem. Co., U.K. Absolute ethanol was supplied by Merck-BDH Co., U.K. Round bottomed screw-cap tubes were fitted with PTFE liners in the caps (7.5 ml, Fisons TKV-173-010H and TKV-178-010A, Fisons Scientific Ltd., Loughborough, Leicestershire, U.K.).

The following method was based on that described by Morrison and Laignelet (1983).

Starch Solubilisation

The starch sample was accurately weighed (40 mg ± 0.1 mg) into a tube. Between 10-20 mg was used for the high AM starches (Hylon V starch and Hylon VII starch). To the pre-weighed tube was added 5 ml UDMSO (90/10%, v/v, DMSO/6M urea), then capped, and immediately mixed vigorously on a vortex mixer. The tube was re-weighed to obtain the weight of the starch-UDMSO solution. The tubes were mixed again and placed in a boiling water bath for 90 min, with intermittent mixing, especially for the first 15 min. Then the tubes were allowed to cool and checked for the absence of clear gel (as precipitates or gelatinous sediments are transparent), before using the solution for subsequent analyses.

Starch Precipitation

A 0.5 ml aliquot of the starch-UDMSO solution was weighed into a fresh screw cap tube and 4.5 ml of absolute ethanol added. The tubes were capped and left to stand for 15 min. The precipitate was centrifuged at 2,620 rpm for 5 min (Chilspin, MSE, Fisons Scientific Ltd., U.K.) and decanted, allowing for a few seconds drainage time. The starch precipitate was redissolved by adding 0.5 ml of UDMSO, capping and mixing. The tubes were subsequently placed in a boiling water bath for 15 min, with intermittent mixing, and then cooled.

Determination of the Total Amylose Contents of Starches

The solutions were rapidly transferred into 50 ml volumetric flasks. About 47 ml of distilled water was added, using the first few millimetres of water to rinse out the tube. The flasks were swirled. To each flask was added 1 ml of I_2/KI solution (0.2/2%, w/v) and swirled immediately. The time required to transfer the UDMSO solution and develop the colour was always less than 60 seconds. The volumes of the solutions in the flasks were made up to 50 ml with distilled water and the flasks were swirled again.

The flasks were left for 15 min and then the temperature of the solution was measured. The absorbances of the solutions were measured at 635 nm (Philips PU 8620 UV/VIS/NIR Spectrophotometer). The absorbance of a blank (distilled water and I_2/KI solution) was subtracted from the absorbances of the samples. The Blue Value was calculated as:

$$\text{Blue Value} = \frac{A635\ nm \times 0.005 \times w_2}{w_1 \times (1-M) \times w_3}$$

where,

w_1 = weight of starch dissolved in 5 ml UDMSO (g)

w_2 = weight of (starch + 5 ml UDMSO) (g)

w_3 = weight of (starch + UDMSO) aliquot for precipitation (g)

M = starch moisture content (%, w/w)

The Blue Value at temperature 't' was adjusted to temperature at 20°C (by using the appropriate correction factor from Table 5.3.1):

$\text{Blue Value}_{(20°C)} = \text{Blue Value}_{(t)} + [\text{correction factor} \times (t-20)]$

The total AM contents of the cereal starches and the *Musa* starches were calculated using the regression equation devised by Morrison and Laignelet (1983) (below). Morrison and Laignelet (1983) used cereal starches with different proportions of AM and AP to devise an equation for the determination of the total AM contents of cereal starches. This was derived from the linear regression of the plot of the Blue Values of the cereal starches (determined by the iodimetric assay) versus their AM contents (determined by 1,6-debranching and GPC).

$$\text{AM content (\%)} = (28.414 \times \text{Blue Value}) - 6.218 \quad (r = 0.9972, n = 34)$$

Table 5.3.1 Correction Factors for Blue Values not measured at 20°C (Morrison and Laignelet, 1983)

Blue Value (BV)	Correction Factor
0.22-1.80	0.0078
2.00	0.0082
2.20	0.0094
2.40	0.0102
2.60	0.0109
2.80	0.0116
3.00	0.0124

5.3.3 Results and Discussion

Total Amylose Contents of the Starches

The AM content of a starch is an important quality parameter. The iodimetric assay was used to estimate the total AM contents of a variety of *Musa* starches which had been isolated from both unripe and ripe fruits, using an equation specifically devised for cereal starches (section 5.3.2). Cereal starches of known AM contents were used for comparison with the *Musa* starches (Table 5.3.2).

In Table 5.3.2, plantain starch and cooking banana starch had higher total AM contents than dessert banana starch, as was seen in the GPC experiments (Table 5.2.2). However, the total AM contents of all of the *Musa* starches, determined by the iodimetric assay, were higher than their AM contents, determined by GPC (Table 5.3.2). According to reports in the literature, the reason for this overestimate is probably because iodine can bind linear AM, the intermediate fraction, and also branched AP (Takeda *et al.*, 1987; Inouchi *et al.*, 1983; Bradbury and Bello, 1993), and unlike the iodimetric assay, the GPC method discounts the intermediate and AP fractions. Therefore, the relationship between BV and AM content is dependent on the structure and molecular characteristics of the starch. However, the total AM contents of the cereal starches were not significantly different to their AM contents, as determined by the suppliers of the cereal starches, or to their AM contents determined by GPC (Table 5.3.2). As the equation devised by Morrison and Laignelet (1983) appeared to be suitable for the determination of the total AM contents of the cereal starches, though unsuitable for the determination of the total AM contents of the *Musa* starches, this suggests that there are structural or molecular differences between the *Musa* starches and the cereal starches. This was demonstrated by the plot of the AM contents of the *Musa* starches versus their BVs (Figure 5.3.1): though there was a linear correlation between these two parameters ($r = 0.831$, Figure 5.3.1), some of the points on the graph deviated from linearity. Morrison and Laignelet (1983)

suggested that problems (or inconsistencies in the results) could arise if the iodimetric assay was used for certain starches if short-chain AM (DP<200) or anomalous AP (λ_{max}> 540 nm) were present, in which case they advise that selective enzymic debranching followed by gel permeation chromatography should be used for the AM content determination of these starches. The DP of the AM fraction of the *Musa* starches was unknown, though the wavelength of maximum absorbance of the AP fraction of the *Musa* starches was about 540 nm (Table 5.2.1). The results suggest that it is better to determine the AM contents of *Musa* starches using enzymic debranching followed by GPC, rather than by iodimetric assay.

Table 5.3.2 Total Amylose Contents of Starches Determined by the Iodimetric Assay (calculated using the cereal equation from Morrison and Laignelet, 1983)

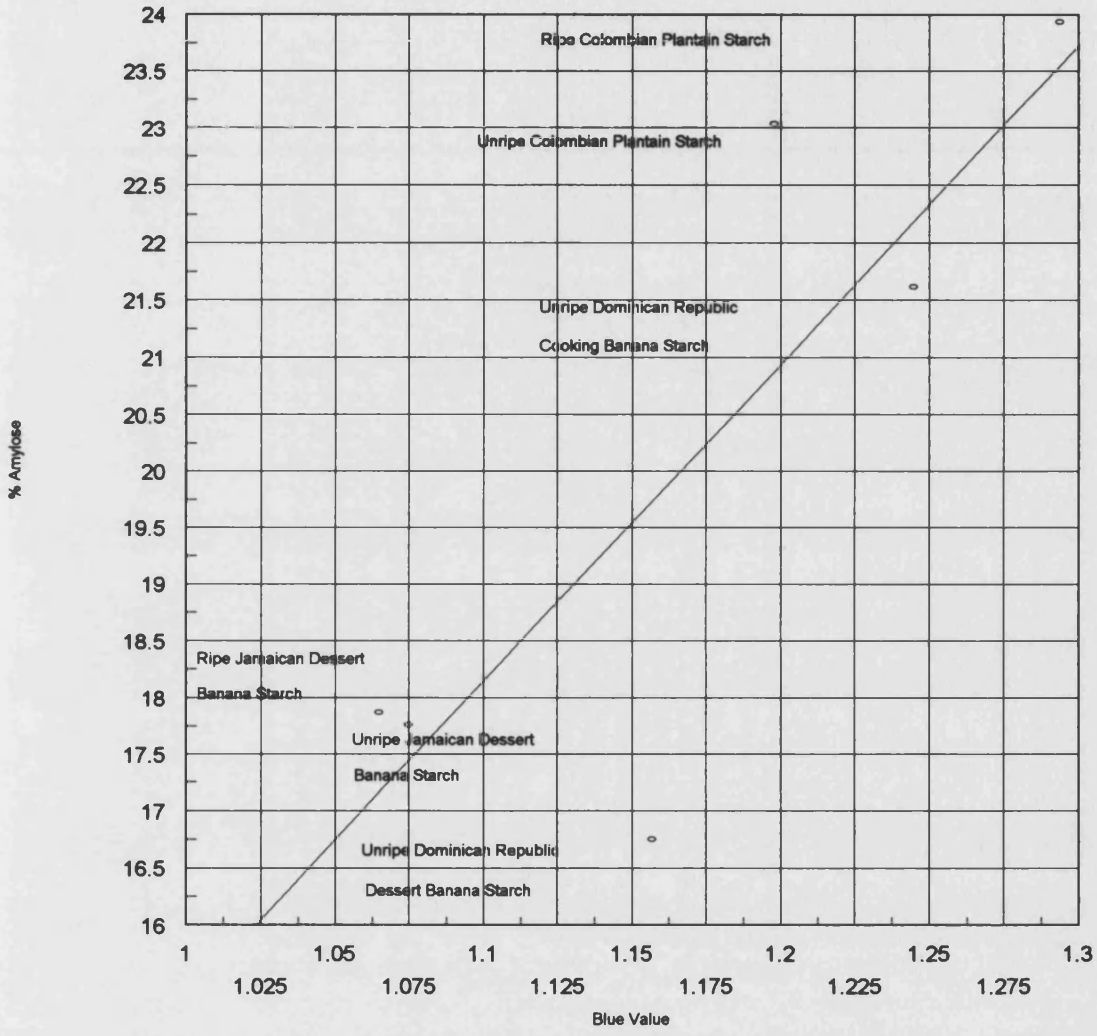
Starch Sample	Moisture Content (%)	Blue Value (BV)	Total AM Content (%)	AM Content (%) (From Table 5.2.2)
Unripe Dominican Republic Dessert Banana (day 1)	12.4	1.2 ± 0.0 (n = 6)	26.7 ± 0.0 (n = 6)	16.8 (n = 3)
Unripe Jamaican Dessert Banana (day 1)	6.9	1.1 ± 0.0 (n = 12)	24.3 ± 1.3 (n = 12)	17.8 (n = 2)
Ripe Jamaican Dessert Banana (day 20)	7.7	1.1 ± 0.0 (n = 6)	24.1 ± 0.4 (n = 6)	17.9 (n = 3)
Unripe Dominican Republic Plantain (day 2)	9.1	1.2 ± 0.0 (n = 6)	27.8 ± 0.2 (n = 6)	ND
Unripe Colombian Plantain (day 1)	5.5	1.3 ± 0.0 (n = 8)	30.6 ± 1.2 (n = 8)	23.0 (n = 4)
Ripe Colombian Plantain (day 18)	8.8	1.2 ± 0.1 (n = 12)	29.2 ± 2.8 (n = 12)	23.9 (n = 3)
Unripe Dominican Republic Cooking Banana (day 3)	8.7	1.2 ± 0.0 (n = 6)	28.6 ± 0.6 (n = 6)	21.6 (n = 3)
Maize (25% AM)*	11.5	1.2 ± 0.0 (n = 18)	27.7 ± 0.8 (n = 18)	25.4 (n = 2)
Waxy Maize (0% AM)*	14.5	0.3 ± 0.0 (n = 24)	1.4 ± 0.3 (n = 24)	1.2 (n = 2)
Hylon V (50% AM)*	14.5	2.2 ± 0.1 (n = 12)	55.5 ± 2.1 (n = 12)	ND
Hylon VII (70% AM)*	12.4	2.7 ± 0.2 (n = 18)	70.8 ± 4.7 (n = 18)	ND

± represents Standard Deviation

* AM content of the starches determined by the suppliers

Figure 5.3.1 Linear Regression of the Relative Amylose Contents of the *Musa* Starches versus their Blue Values

The AM contents of the *Musa* starches, determined by GPC (Table 5.2.2), were plotted against their BVs, determined by the iodimetric assay (Table 5.3.2).



Regression coefficient, $r = 0.831$, Number of points = 6, b = 27.914, and a = -12.561, where, $y = a + bx$

5.4 Conclusions

- Plantain starch and dessert banana starch both displayed C-type X-ray diffraction patterns. This indicated that the structural arrangements of the starch polymers within the starch granules of the different *Musa* types were similar. The structural arrangement of the starch polymers within the waxy maize starch granules (A-type crystalline pattern) and the potato starch granules (B-type crystalline pattern) were different to each other and to the *Musa* starches.

- The crystallinities of unripe plantain starch and the unripe and ripe dessert banana starches were similar. The crystallinities of the *Musa* starches increased with increasing ripeness of the fruits from which the starches were isolated as a result of enzymic degradation of the starch polymers and re-association of the polymer chains, which increased the structural order within the ripe starch granules. This increase was higher for plantain than for dessert banana.

- Plantain starch had a similar AM content to cooking banana starch, which was slightly higher than that of the dessert banana starches, determined by GPC. The total AM contents of the starches were determined using the iodimetric assay (a colorimetric technique), which relied on the binding of an I_2-KI solution to the AM component. The cereal equation devised by Morrison and Laignelet (1983) was used to calculate the total AM contents of the starches. The iodimetric assay overestimated the AM contents of the *Musa* starches compared to the GPC data, though the total AM contents of the cereal starches were not significantly different to their AM contents determined by GPC (Table 5.3.2). The results suggest possible structural and molecular differences between the cereal and *Musa* starches. Due to the differences in the results of the AM contents of the starches between the two techniques, it is suggested that the GPC technique is

probably a better technique for the determination of the composition of AM in *Musa* starches.

- Overall, the results of the structural and molecular characteristics of the different *Musa* starches were generally similar, and there did not appear to be any distinguishable differences between the *Musa* types. There were differences in the structural arrangements of the polymers within the starch granules between the different species of starch (*i.e. Musa* starch, cereal starch and potato starch), though the molecular characteristics of the AP component of the *Musa*, cereal and potato starches were similar (Tables 5.2.3 to 5.2.6). However, there were differences in the extent of branching of the AP fractions (Fr. III to Fr. II ratio) between the starches: the *Musa* starches had a similar amount of branching in their AP components (ranging from 1.5: 1 to 1.6: 1), which was lower than that of the cereal starches, but higher than that of the potato starch. It is suggested that as GPC is not a very sensitive technique, minor differences in the molecular characteristics of the AP components between the *Musa* starches would not have been detected. It is proposed that further research using more sensitive techniques than GPC (see Chapter 8) is needed if any significant molecular differences between the different *Musa* types are to be found.

Chapter 6

Pasting Characteristics of *Musa* Starches and Flours

6.1 Introduction

In this study, the Brabender Viscoamylograph and the Rapid Viscoanalyser (see also section 2.3.6) were used to demonstrate and compare the cooking behaviour of flours and starches from different *Musa* types at different stages of fruit maturity. Little research has been reported previously on the cooking properties of *Musa* starches or flours using the Rapid Viscoanalyser.

6.1.1 Uses of the Brabender Viscoamylograph and the Rapid Viscoanalyser

The Brabender Viscoamylograph and the Rapid Viscoanalyser are rotational viscometers used for research, development and product control in the food industry as well as in the chemical, paper, and textile industries. The Brabender Viscoamylograph and the Rapid Viscoanalyser both measure the viscosity changes which occur in a starch or flour suspension during a programmed heating and cooling cycle. The heating and cooling cycle is varied in order to simulate the processing conditions for cooking a starchy food. In the food industry they are useful tools for studying the pasting properties of starches and the effects of other food ingredients on starch performance (*e.g.* see Deffenbaugh and Walker, 1989a).

The combination of robust construction and precise results in connection with its simple use has made the Brabender Viscoamylograph a long approved and standard instrument in the starch industry. The Rapid Viscoanalyser is a more recent development and was originally developed for the Australian wheat industry as a quick test for sprout damage (Walker *et al.*, 1988; Ross *et al.*, 1987). The Rapid Viscoanalyser produces pasting patterns similar to those of the Brabender Viscoamylograph. The advantages of the Rapid Viscoanalyser compared to the Brabender Viscoamylograph for the analysis of starch cooking properties include smaller sample size (~3 g), rapid analysis time (as little as 5 to 12 minutes per sample), and time or temperature versatility. Therefore, the Rapid

Viscoanalyser has opportunities for uses in research, plant breeding, or product development to screen large numbers of samples preparatory to longer tests. In the experiments described in this chapter, the Brabender Viscoamylograph was used to demonstrate the pasting properties of *Musa* flours and the Rapid Viscoanalyser was used to investigate the pasting properties of *Musa* starches and a *Musa* flour sample. There was insufficient starch isolated from the *Musa* fruits (according to section 3.2.2) to be able to perform Brabender viscoamylograms of the *Musa* starches.

6.1.2 The Brabender Viscoamylograph

The Brabender Viscoamylograph consists of a stainless steel cup with eight small vertical rods or pins in circular rows inside the cup. The cup is rotated at 75 rpm and heated by radiant heat from electrical spirals outside the cup. A cooling probe is immersed in the flour or starch suspension in the cup. A thermoregulator controls the heating and cooling elements so that the desired rate of change in temperature is achieved (Dempster, 1984).

The circular lid covering the rotating vessel has seven small vertical rods or pins which extend into the flour or starch suspension. A torsion spring is connected to the circular lid via a vertical shaft. When the cup is rotated the pins stir the suspension and the torque of the suspension against them turns the shaft until it is balanced by the torsion spring. The viscosity changes of the suspension are recorded on a chart by a pen which is attached to the torsion spring. Viscosity is expressed in terms of 'Brabender Units' (BU).

6.1.3 The Rapid Viscoanalyser

The Rapid Viscoanalyser is a simple box into which is placed a Rapid Viscoanalyser aluminium can containing the starch- or flour-water suspension together with a plastic paddle. The Rapid Viscoanalyser mixes the suspension by operating the paddle and takes the suspension through a programmed heating and cooling cycle. The resistance of the

paddle to increases and decreases in the viscosity of the cooked and cooled starch or flour suspension is recorded on computer software. The viscosity is measured in terms of 'Stirring Number Units' (SNU).

6.1.4 Pasting Profiles

Pasting characteristics refer to the changes in viscosity in an aqueous flour or starch suspension during changes in temperature. The consistency of the starch or flour paste (referred to as viscosity) throughout the entire cooking and cooling process is derived from the Brabender viscoamylogram or the Rapid viscogram pasting profile. There are six important points on a pasting profile which relate to the changes in the starch granules during cooking and cooling as described below. The abbreviations used for each of the six points on the Brabender viscoamylograms and Rapid viscograms are based on those used by Dempster (1984).

6.1.4.1 Pasting Temperature (T^1)

During a typical analysis, the temperature increases from room temperature to 50°C, and from then on rises at a rate of 1.5°C/min. The pasting temperature (T^1) is taken as the point of initial inflection of the trace and it indicates that the starch granules have begun to swell and gelatinise, thereby increasing the viscosity sufficiently to be recorded. In the aqueous starch or flour suspension, the function of the water molecules is to plasticise the amorphous regions of the starch granules during gelatinisation (Deffenbaugh and Walker, 1989a), and the extent of starch pasting is a function of the availability of water to the starch granules. Swelling begins in the least organised, amorphous inter-crystalline regions of the granule and as this phase swells, it exerts a tension on the neighbouring crystallites and tends to distort them (Hari *et al.*, 1989). The 'gelatinisation' (or pasting) process is a forcing apart of the groups of glucose chains, with relief of internal stress due to dissociation of the relatively weak bonding, and thereby the loss of crystalline structure

(as seen by the disappearance of birefringence under the optical light microscope using polarised light), and swelling of the granule (Leach *et al.*, 1959; Caesar, 1932). According to the definition of 'pasting temperature' described by Atwell *et al.* (1988), starch granules begin to paste before the gelatinisation temperature is reached.

6.1.4.2 Peak Viscosity (PV)

Further heating above the pasting temperature leads to the uncoiling or dissociation of the double helical regions in the starch granules and break up of the AP crystallite structure. Increased molecular mobility with further hydration permits a redistribution of molecules within the starch granules and the smaller AM molecules diffuse out (Hari *et al.*, 1989). This swelling and solubilising of the starch granules continues with heating until a maximum peak viscosity (PV) is attained. Miller *et al.* (1973) found that the increase in the viscosity of a wheat starch suspension heated in an excess of water was mainly due to the release of exudate from the granules, rather than the granular swelling, and they suggested that solubilised AM contributed to the PV. This finding was also confirmed by Ghiasi *et al.* (1982).

6.1.4.3 Viscosity of the Suspension at 95°C (HV^1)

The viscosity of the suspension is measured at 95°C (HV^1), which is the highest temperature the viscosity of the suspension can be conveniently assessed. The PV in relation to the viscosity of the suspension at 95°C (HV^1), indicates how easily the starch can be 'cooked', *i.e.* the relation between these two points on the pasting profile show the temperature (and time) when the swollen starch granules break down.

6.1.4.4 Viscosity of the Suspension after 20 min at 95°C (HV^2)

With continued heating, the solubility of the granules increases and the integrity of the granules eventually breaks down, primarily due to the loosening and collapse of the intermolecular forces between the AM molecules. Thus, the viscosity of the suspension decreases during the 95°C holding period (HV^2) due to the breakdown of these forces.

6.1.4.5 Viscosity of the Cooled Suspension at 50°C (CV^1)

Retrogradation ('set-back') is shown by an increase in the viscosity of the suspension on cooling (CV^1). The retrogradation tendency of the starch is largely determined by the affinity of hydroxyl groups in one molecule for another, which occurs mainly between the AM molecules. Hydrogen-bonding between parallel AM chains forms a stable gel network of low solubility (but branching hinders association), and the essentially linear AM molecules which form the stable cooled paste have an optimum chain length of 100-200 anhydro-glucose units (Rickard *et al.*, 1991; Kruger and Murray, 1976; Swinkels, 1985). Larger molecules associate less readily than molecules of this optimum intermediate chain length, whereas very small molecules undergo rapid Brownian motion which can have a disordering effect (Collison, 1968a). Other factors besides the molecular weight of the AM component which can have an effect on the retrogradation rate of a starch or flour suspension include the concentration of AM, the concentration of AP, the temperature, the pH, and non-starch components in the medium (Swinkels, 1985).

6.1.4.6 Viscosity of the Cooled Paste after 20 min at 50°C (CV^2)

After holding at 50°C for 20 min (CV^2), the viscosity represents the stability of the cooled gel to continued stirring, *i.e.* the strength of the bonding between the aggregated starch molecules.

6.2 Materials and Methods

The *Musa* fruits comprised unripe Dominican Republic dessert bananas (from day 2 after the fruits were purchased, peel colour score 2 to 3, section 3.2.1); unripe and ripe Jamaican dessert bananas (from day 1 and day 18, peel colour score 1 to 2, and 6, respectively); unripe and ripe Colombian plantains (from day 1 and day 18, respectively); unripe Dominican Republic plantains (from day 2); and unripe Dominican Republic cooking bananas (from day 2). Maize starch and waxy maize starch were used as reference materials.

Flour and Starch Preparation and Analysis of the Flour Samples

Starch isolations and flour preparations were made using different *Musa* types (above) according to sections 3.2.2 and 3.2.3, respectively. The starch contents of the flour samples were determined using the glucose oxidase method (section 3.2.5). All of the *Musa* flour samples and the reference samples (maize starch and waxy maize starch) were refluxed with ethanol (section 3.2.6), prior to their use in the Brabender Viscoamylograph and Rapid Viscoanalyser experiments. In addition, the moisture contents of the starch and flour samples were determined (section 3.2.4) in order to calculate the weight of sample required in either the Brabender Viscoamylograph or Rapid Viscoanalyser flour-water or starch-water suspensions.

6.2.1 Brabender Viscoamylograph Procedure

A calibrated Brabender Viscoamylograph PT-100 (Duisburg, West Germany) was equipped with either a 700 cm g sensitivity cartridge or a 250 cm g sensitivity cartridge. When the Brabender Viscoamylograph was equipped with a 250 cm g sensitivity cartridge a correction factor was used to convert the pasting profile values into those which would have been obtained with a 700 cm g sensitivity cartridge (Clive Knibb, pers. comm.):

$$\text{Sensitivity Cartridge Correction Factor} = \frac{250}{700} = 0.4$$

The sample (~20-25 g) was weighed in a 250 ml beaker. 150 ml of distilled water was poured onto the sample and mixed well (with a Black and Decker hand whisk) to form a lump-free suspension. The suspension was poured into the Brabender Viscoamylograph bowl and the remaining volume of water required to make up a 5% suspension was used to rinse out the beaker and hand whisk into the Brabender Viscoamylograph bowl. The measuring sensor and cooling probe were attached and lowered into the suspension in the bowl. The Brabender Viscoamylograph pasting programme was started. The starting temperature was 50°C rising by 1.5°C/min to 95°C. The suspension was maintained at 95°C for 20 min. The suspension was cooled to 50°C and was held at this temperature for 20 min. 5% *Musa* flour suspensions and 5% cereal starch suspensions were used in the experiments.

Data handling

The six important points on the Brabender viscoamylograms (see Appendix II) were plotted on a computer graphics software package, for ease of comparison with the Rapid viscograms.

6.2.2 Rapid Viscoanalyser Procedure

2 to 4 g of the starch or flour sample (of known moisture content) was weighed into a Rapid Viscoanalyser aluminium can. Deionised water was added so that the final weight of the contents of the can was 25 g. A 10% flour suspension and 8% starch suspensions were used. The mixture was stirred by hand with a Rapid Viscoanalyser plastic paddle which was then inserted into the can. The can was placed in a calibrated Rapid

Viscoanalyser RVA-3CR (Newport Scientific Ltd., Narrabeen, N.S.W., Australia) and automatically clamped tight by a split copper block which was pre-heated to the desired temperature (50°C) and the cycle started. The paddle automatically started spinning at 900 rpm for seven seconds, and then slowed to a constant 160 rpm for the remainder of the test. As the paste thickened the power required to turn the motor at constant speed increased and was recorded using a computer logging system. The temperature profile was the same as for the Brabender Viscoamylograph, *i.e.* 50°C rising by 1.5°C/min to 95°C, where it was held for 20 min. The suspension was subsequently cooled to 50°C and maintained at this temperature for 20 min.

6.3 Results and Discussion

The main objective of the experiments described in this chapter was to examine the pasting characteristics of flours and starches from different *Musa* fruits, highlighting the differences between the different *Musa* types. Starch is known to affect the cooking quality of plant products. Plantains and cooking bananas are mostly cooked before consumption and are often processed into flours before they are cooked (see section 2.2.4). In this study, the pasting properties of plantain and cooking banana flours were examined. Dessert bananas, however, are mostly eaten raw when ripe. Flour was produced from dessert bananas and was also used in the experiments as another comparative *Musa* type. As starch is the main component of unripe *Musa* fruits, and also the main component of the flours produced from unripe *Musa* fruits, it is the starch and the characteristics of the starch granules which are mainly responsible for the cooking qualities of the unripe *Musa* flours. The behaviour of unripe *Musa* flours and starches during cooking should also be similar. As there was insufficient starch isolated from the *Musa* fruits (around 5-10 g), the Rapid Viscoanalyser was the preferred technique for measuring the pasting characteristics of these *Musa* starches, whereas the *Musa* flours could be used in the Brabender Viscoamylograph.

The pasting behaviour of unripe and ripe *Musa* flour and starch samples within the same and between different *Musa* types were compared. The two main *Musa* types used in the experiments were plantain and dessert banana. A cooking banana flour suspension was also studied. Due to insufficient sample material, it was not possible to examine the pasting characteristics of ripe cooking banana flour. The pasting characteristics of the flour suspensions are shown in Table 6.3.1, and the pasting characteristics of the starch suspensions are shown in Table 6.3.2.

The Reference Starches

The AM content is considered to be very important for cooking characteristics of starches (Berríos and González, 1971). As maize starch has a high AM content (25%) compared to waxy maize starch (0-2%), these two starches are useful when considering the relationship between the measured pasting characteristics and the AM content of the starch granules in the suspension. Maize starch and waxy maize starch were run in addition to the *Musa* starches (and flour sample) using the Rapid Viscoanalyser (see Figure 6.3.7).

Pasting Profile Differences Between the Two Techniques

The pasting characteristics of the starches and flours were obtained using two different instruments. The Brabender Viscoamylograph was used to examine the pasting properties of the *Musa* flours and the Rapid Viscoanalyser was used to examine the *Musa* starches and the cereal starches. The pasting behaviour of a *Musa* flour sample was also investigated using the Rapid Viscoanalyser, which illustrated that there were pasting profile differences between the two techniques (Figure 6.3.1).

The instruments and techniques are detailed at the beginning of this chapter. It is important to appreciate the differences between the instruments, as this has some bearing on the comparison of the pasting characteristics (temperatures and viscosities) between the two techniques. Key differences between the instruments include the fact that the Rapid Viscoanalyser has more rapid stirring than the Brabender Viscoamylograph (160 rpm compared to 75 rpm, respectively), such that the Rapid Viscoanalyser exerts a greater shear on the starch or flour suspension. In addition, the Rapid Viscoanalyser uses a smaller sample size, so that even though the temperature profile is the same, the heating and cooling rates may differ. Similarly, Deffenbaugh and Walker (1989b) suggested that

the instruments were not inter-changeable, and the results for the two instruments were less similar for flour than for starch samples.

The different suspension concentrations used in the Rapid Viscoanalyser and Brabender Viscoamylograph affected the pasting characteristics. For example, in Figure 6.3.1, the concentration of flour in the suspension was higher in the Rapid Viscoanalyser (and therefore, it had a higher concentration of starch) than that in the Brabender Viscoamylograph. This was the possible cause of the pasting temperature of the Rapid viscogram being lower than that of the Brabender viscoamylogram. The relationship between the pasting temperature and the concentration of starch in the suspension has also been reported by Collison (1968b).

***Musa* Flour Suspensions Versus *Musa* Starch Suspensions**

When considering the results, it is important to consider that where the Brabender Viscoamylograph was used to compare *Musa* flours, the Rapid Viscoanalyser was mainly used to compare *Musa* starches. The additional components present in the *Musa* flours may affect the pasting characteristics: in Figure 6.3.2, the non-starch components in the unripe Colombian plantain flour appeared to affect the pasting temperature and the overall swelling profile, though the viscosity of the suspension upon retrogradation did not appear to be significantly affected. It is interesting to consider all of the other components present in the flour samples. Suntharalingam and Ravindran (1993) investigated the composition of green cooking banana flour: the average chemical composition comprised crude protein (3.2%), crude fat (1.3%), ash (3.7%), neutral detergent fibre (8.9%), acid detergent fibre (3.8%), cellulose (3.1%), lignin (1.0%), hemicellulose (5.0%); the carbohydrate composition comprised soluble sugars (2.8%), starch (70%), and other non-starch polysaccharides (12%).

Origins of the *Musa* Types

The *Musa* samples were prepared from fruits from a variety of origins, which were probably grown under different agronomic conditions. This may also have increased the variability between the pasting characteristics. Therefore, the distinction between the different *Musa* types is less clear than it might have been had all of the fruits been obtained from the same location. However, Figure 6.3.3 shows the pasting profiles of flours made from three different *Musa* types all of which had been grown in the Dominican Republic. Thus, the main differences between the pasting characteristics of the different flour suspensions in Figure 6.3.3 were probably *Musa* type dependent (see below).

Differences in the Pasting Characteristics Between the Different *Musa* Types

In Tables 6.3.1 and 6.3.2, differences were seen in the pasting characteristics between the different *Musa* types. Though the pasting temperatures of the different *Musa* types were similar, the unripe dessert banana flour suspensions had higher PVs than the unripe plantain flour and unripe cooking banana flour suspensions (Table 6.3.1). The PV of the unripe dessert banana starch suspension was also higher than that of the unripe plantain starch suspension (Table 6.3.2). Kayisu *et al.* (1981) observed that banana starch had a high degree of solubilisation which was intermediate between that of potato and tapioca starches. The degree of starch solubilisation was explained by the extent to which the AM was engaged in the crystalline or micellar regions of the granule and its leaching from the granule. A higher solubility suggested a lower involvement of AM in the crystalline regions and *vice versa* (Kayisu *et al.*, 1981). Using these observations reported by Kayisu *et al.* (1981) together with the results of the experiments described in this chapter, it is proposed that the unripe dessert banana suspensions had higher PVs than the other unripe *Musa* suspensions because the AM component in the unripe dessert banana starch granules was possibly less tightly bound to the AP component than in the other *Musa* types, *i.e.* more soluble AM was exuded by the unripe dessert banana starch granules than

the unripe plantain and unripe cooking banana starch granules as a result of differences in the molecular associations of the components within the starch granules.

The unripe dessert banana starch suspension produced a more viscous (*i.e.* more stable) suspension upon retrogradation than the unripe plantain starch suspension (Table 6.3.2). This difference was less clear for the unripe *Musa* flour suspensions (Table 6.3.1). However, the viscosity of the unripe cooking banana flour suspension was lower than the unripe dessert banana and unripe plantain suspensions during retrogradation (Table 6.3.1).

Unripe *Musa* Samples Versus Ripe *Musa* Samples

The *Musa* samples were prepared from both unripe and ripe fruits. The extent of fruit maturity post-harvest to the day of purchase of the fruits was assumed to be roughly the same for all of the *Musa* types. The day the fruits were purchased was designated as 'day one'. The peel colour of the fruits was subjectively evaluated and, in the experiments, the fruits were described as being either 'unripe' when the colour of the banana peel was colour score index 1 to 3, or 'ripe' when the colour of the banana peel was colour score index 6 or more (see section 3.2.1).

The starch contents of the *Musa* flours decreased as the fruits from which the flours were made became more ripe (Table 6.3.1), though this difference was not significant. Higher starch contents than those in Table 6.3.1 have been reported in the literature which suggests that these researchers were able to use fruits which may have been freshly harvested: Rahman (1963) reported the starch content of flour made from green plantain fruits to be 71.62%; Kayisu *et al.* (1981) reported the starch content of unripe (green) banana flour to be $78.0 \pm 0.4\%$ ($n \geq 2$).

In general, during ripening, the pasting characteristics of the *Musa* starch and flour suspensions changed in a consistent way: the time to reach PV decreased, which indicated that the samples became 'easier to cook' (*i.e.* the time between HV[1] and PV decreased); the stability of the starch granules during continued heating decreased (*i.e.* the viscosity difference between PV and HV[2] increased); and the extent of retrogradation decreased, which suggested that there was less H-bonding between the linear AM molecules as a result of enzymic breakdown of the AM component (*i.e.* the chain lengths of the AM molecules were less than optimum for H-bonds to form) (Tables 6.3.1 and 6.3.2).

Other Factors to Consider

The *Musa* flours used in these studies had been refluxed with 80% ethanol (*i.e.* defatted, see sections 3.2.5.1 and 3.2.6). This procedure could account for some of the differences between the results presented here and those in the literature. Lipid complexed with the AM component in starch granules can restrict the swelling of the starch (Swinkels, 1985). Therefore, the effect of the ethanol-reflux procedure on the pasting profiles would have been to increase the viscosities of the flour suspensions, because lipid or other interfering alcohol soluble compounds had been removed. In the literature, the lipid content of green banana starch and fresh banana pulp has been reported as only 0.2% and 0.12%, respectively (Kayisu *et al.*, 1981; Forsyth, 1980). Thus, as the lipid content of *Musa* flours and starches is so low, this would suggest that the lipid would not have had a pronounced effect on granule swelling.

Relationship Between the Pasting Characteristics and the AM Contents of the Starches

The results of the experiments presented in this chapter were considered on the basis as to whether they could be explained by the differences in the AM contents of the starches. Maize starch has a high AM content and that of waxy maize starch is low, and these cereal

starches were used in the pasting experiments to compare with the behaviour of the *Musa* starches and flours. In the literature, it has been reported that the AM content of dessert banana starch is lower than that of plantain (see section 2.3.3.1). This was found to be the case in Chapter 5, where it was found that the unripe dessert banana starch granules had a lower AM content than the unripe plantain and unripe cooking banana starch granules. As a high AM content can lead to strong H-bonds within the starch granules, this can restrict the swelling of starch granules (Goering and Schuh, 1967). Therefore, a high AM content would be expected to be positively correlated with a high pasting temperature. The results of the maize starch and waxy maize starch suspensions were consistent with this. However, this relationship was not apparent with the *Musa* starch and flour suspensions. The association and arrangements of the AM and AP components in relation to each other, and their possible variations within the starch granule are still matters of debate. It is also possible that the molecular size of the AM component might be a contributing factor in the differences in the pasting temperatures between different starches.

The low AM content of the waxy maize starch granules caused the suspension to produce a high and early PV, whereas the maize starch suspension (high granular AM content) produced a low PV. The PVs of the *Musa* flour and starch suspensions did not correlate consistently with the AM contents of their starch granules. These results suggest that it is possible that the association of the polymer components within the starch granules, rather than their composition, played a more important role in the pasting behaviour of the starches.

In the Rapid Viscoanalyser, the PV was attained at the lowest temperature by the waxy maize starch suspension because there were no AM molecules in the intermicellar regions supporting the crystalline AP regions in the starch granules (Table 6.3.2). Similarly, after 20 min at 95°C (HV^2), the waxy maize starch suspension was the least viscous (*i.e.* most

unstable) compared to the other starch suspensions due to the low AM content of its starch granules (Table 6.3.2).

There was a relationship between the AM content of the starch granules and the viscosity of the retrograded paste for the cereal starches: the waxy maize starch suspension had a low CV^1 and CV^2, as there was very little AM (and therefore few AM molecules) to H-bond with neighbouring chains and form a stable crystalline network, while the maize starch suspension had a high CV^1 and CV^2, which was probably due to the higher AM content of the maize starch granules (Table 6.3.2). However, this relationship for the *Musa* starch and flour suspensions was less clear. The results indicated that during retrogradation, there was less opportunity for H-bonds to form between the linear AM molecules of the unripe cooking banana flour cooled paste as the chain lengths of the AM molecules were less than optimum and were possibly shorter, or more branched, than those of the unripe plantain and unripe dessert banana starch granules. Thus, the retrogradation behaviour of the *Musa* starches may suggest important differences in the molecular characteristics of the AM components between the different *Musa* types, *e.g.* in the chain lengths, or the degree of branching of AM, or both.

Strengths of Bonds Within the Granules

Unlike that of the cereal starches, the comparison between the results of the pasting characteristics of the *Musa* starch and flour suspensions with the AM contents of their starch granules was inconclusive. A relationship between the AM contents of the *Musa* starches and their Rapid viscogram pasting characteristics was only apparent at CV^1 and CV^2, though this relationship was not entirely consistent for all of the unripe and ripe *Musa* types. It has been suggested that the differences in swelling and solubilisation behaviour, as monitored by the Brabender Viscoamylograph and Rapid Viscoanalyser, reflect variations in the strength of non-covalent bonding between the macromolecules

within the starch granule (Asaoka *et al.*, 1992). In addition, Brabender viscoamylograms of *Musa* starches have been reported as showing restricted-swelling with stability (*e.g.* Eggleston *et al.*, 1992; Lii *et al.*, 1982), which is indicative of strong or extensive H-bonding within the starch granules. Consideration of the reports in the literature, and the results of the pasting experiments of the *Musa* starches and flours described in this chapter, would suggest that the extent and strengths of the intra- and inter-molecular bonds within the starch granules, and the molecular characteristics of the AM component, appear to be more important in pasting than the compositions of AM and AP, *i.e.* the AM content did not have a direct bearing on the overall pasting profile.

Figure 6.3.1 Brabender Viscoamylogram and Rapid Viscogram of Unripe Colombian Plantain Flour (day 1)

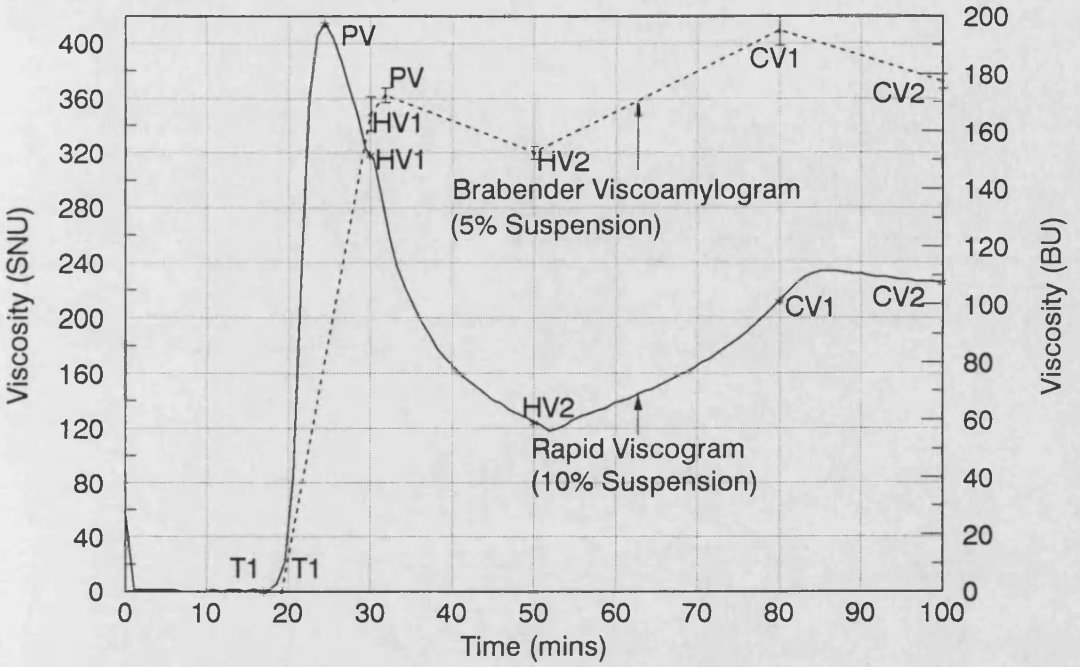
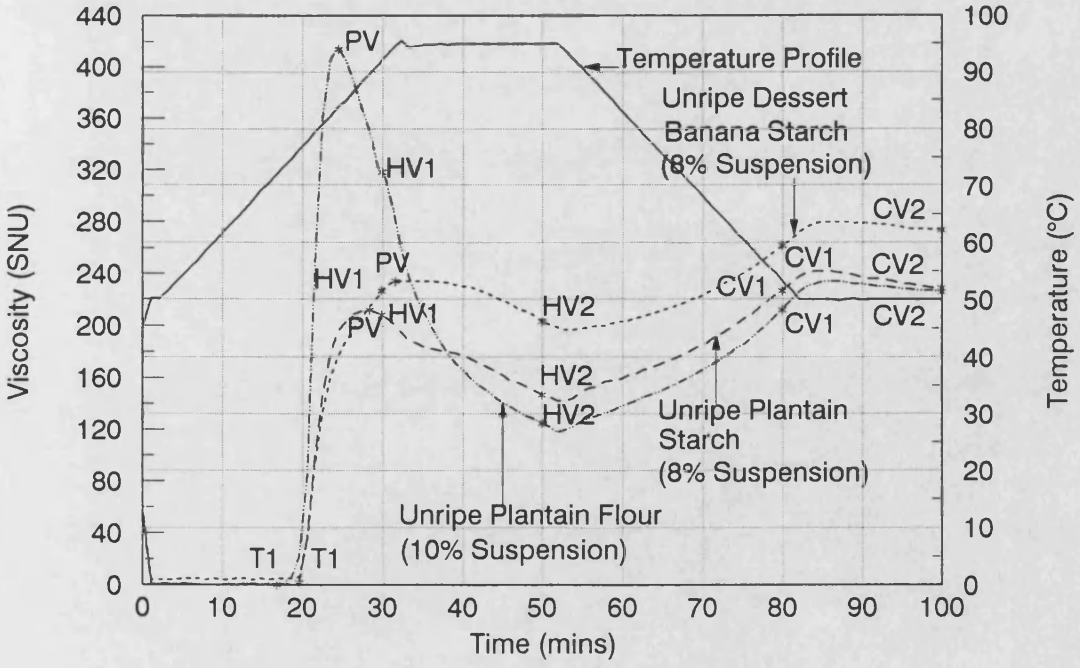


Figure 6.3.2 Rapid Viscograms of Unripe Colombian Plantain Flour, Unripe Colombian Plantain Starch and Unripe Jamaican Dessert Banana Starch (day 1)



Vertical lines indicate Standard Error of the Means

Figure 6.3.3 Brabender Viscoamylograms of Flours from Unripe Dessert Bananas, Unripe Plantains and Unripe Cooking Bananas Grown in the Dominican Republic (all day 2)

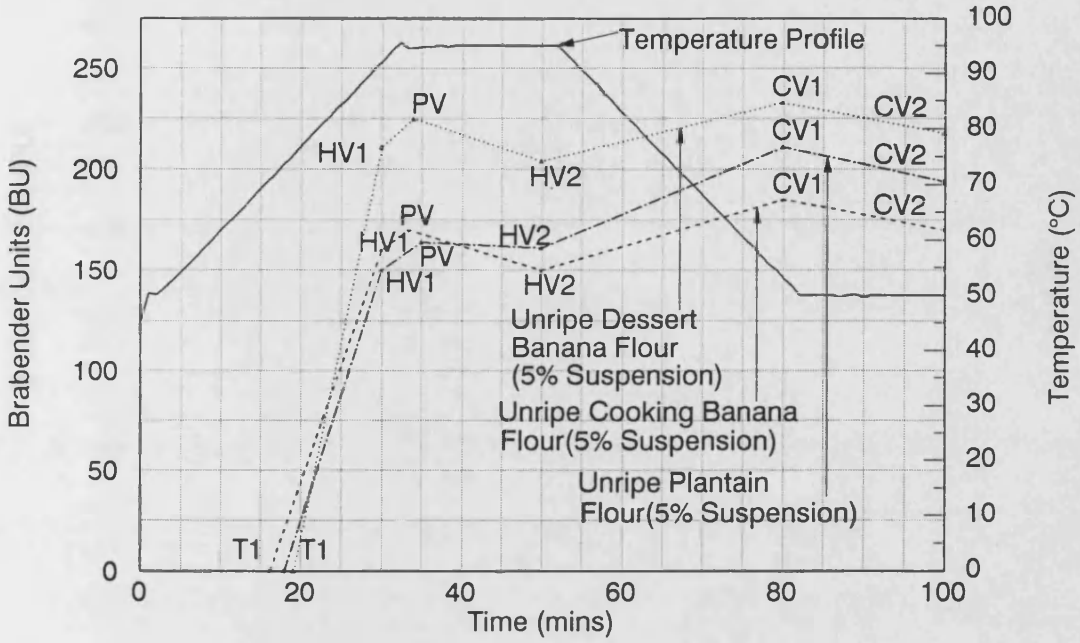
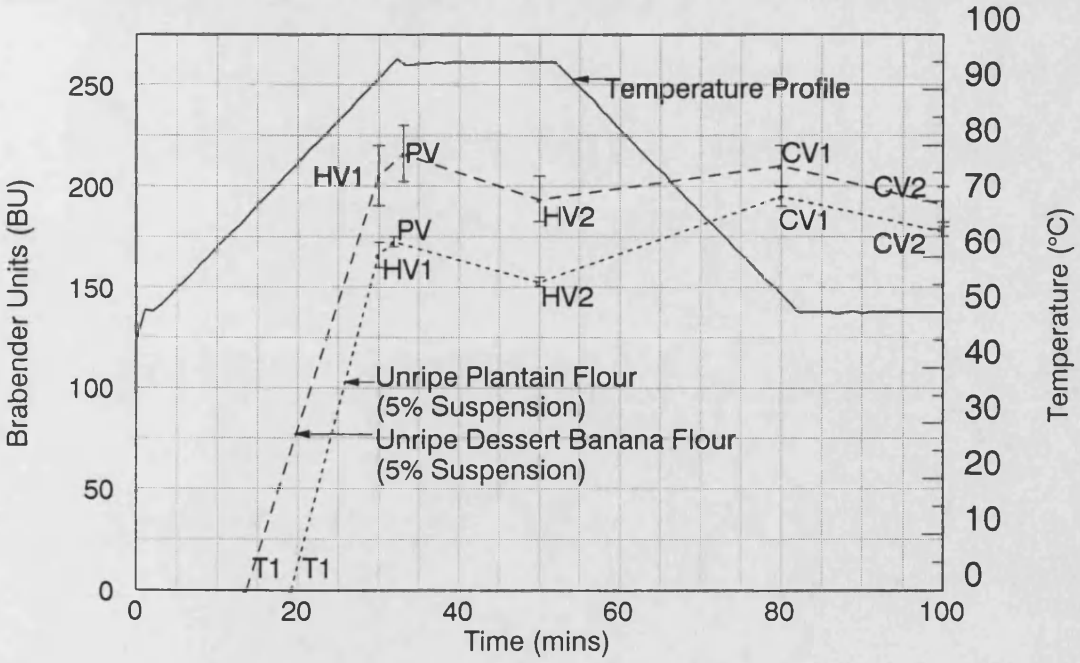


Figure 6.3.4 Brabender Viscoamylograms of Unripe Colombian Plantain Flour and Unripe Jamaican Dessert Banana Flour (both day 1)



Vertical lines indicate Standard Error of the Means

Figure 6.3.5 Brabender Viscoamylograms of Ripe Colombian Plantain Flour and Ripe Jamaican Dessert Banana Flours (both day 18)

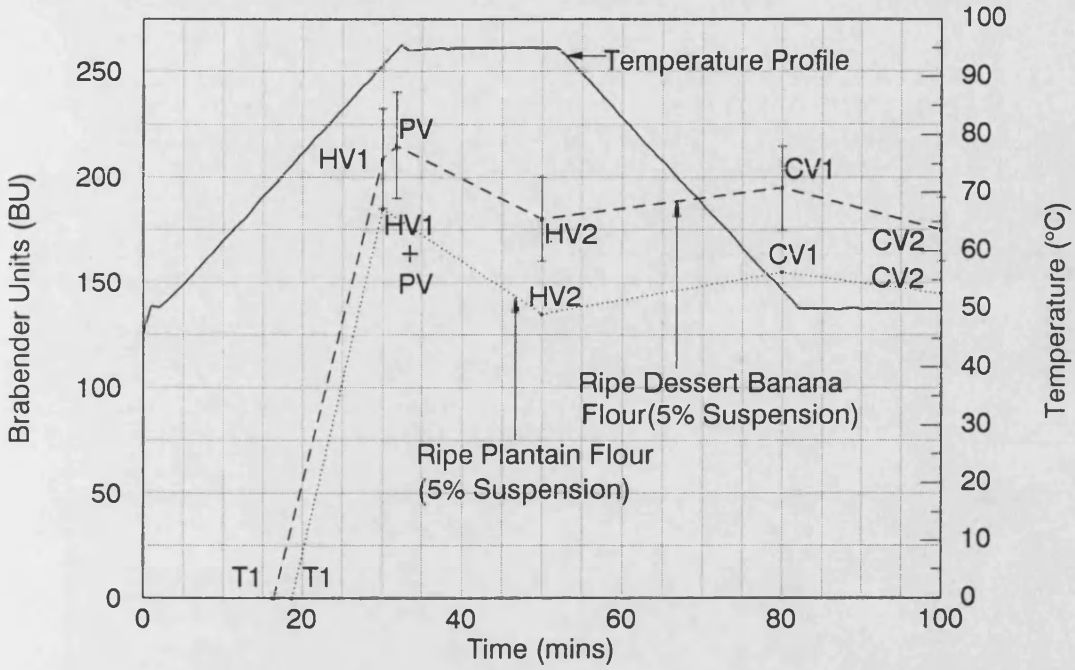
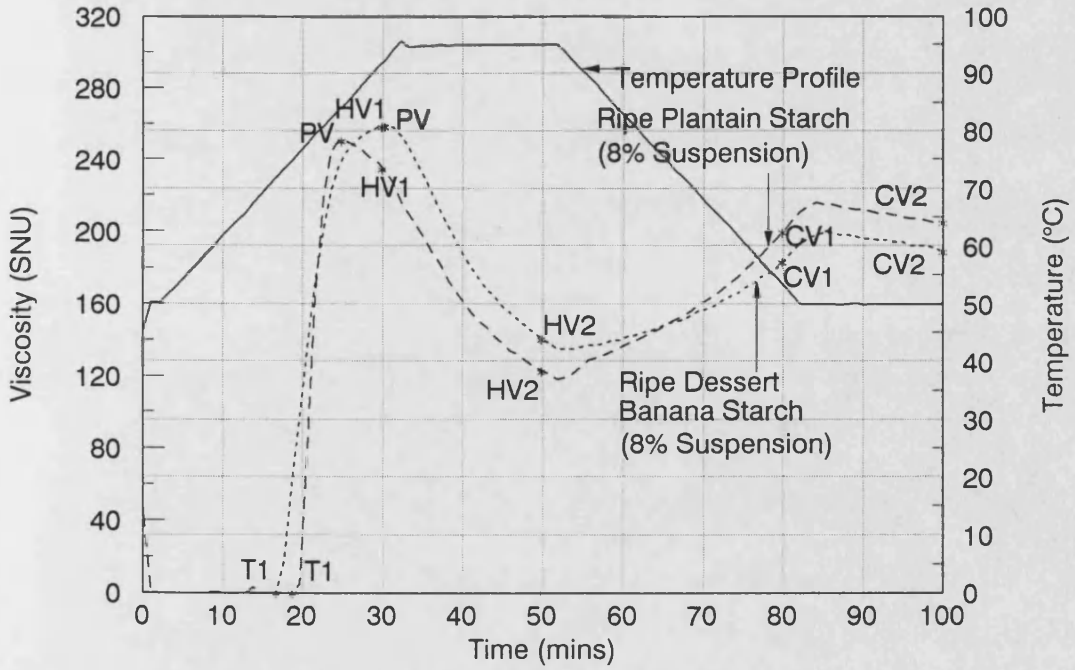


Figure 6.3.6 Rapid Viscograms of Ripe Colombian Plantain Starch and Ripe Jamaican Dessert Banana Starch (both day 18)



Vertical lines indicate Standard Error of the Means

Figure 6.3.7 Rapid Viscograms of Maize Starch and Waxy Maize Starch

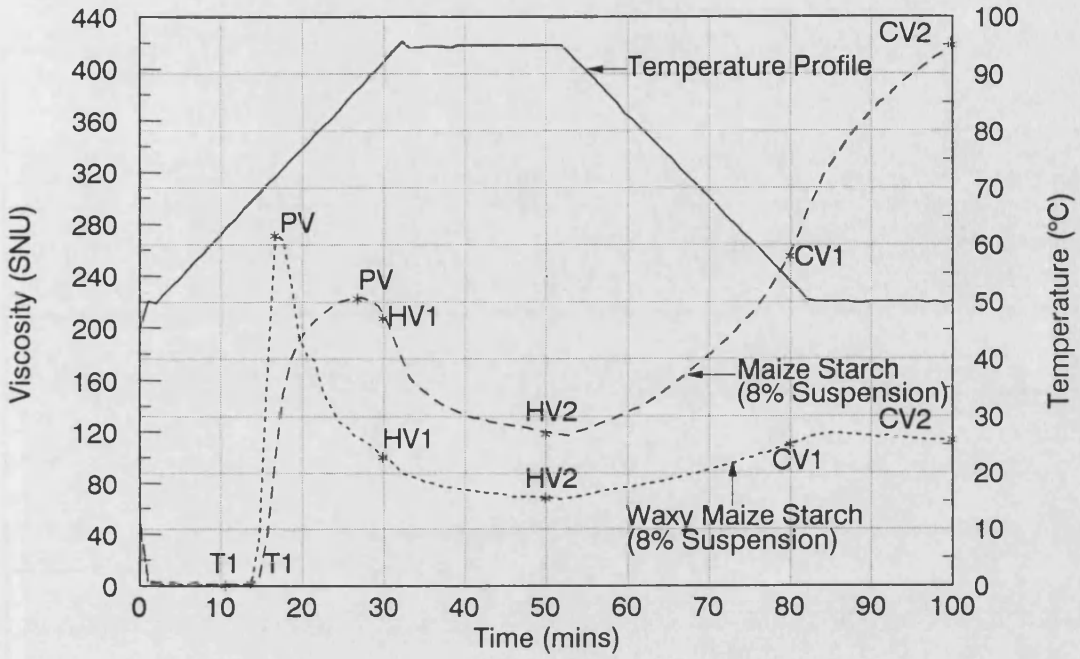


Table 6.3.1 Pasting Characteristics of Brabender Viscoamylograms of Flours from Unripe and Ripe *Musa* Fruits (See Figures 6.3.1 and 6.3.3 to 6.3.5)

Sample	Starch (%) (d.w.b.)	T^1 (°C)	PV (BU)	HV^1 (BU)	HV^2 (BU)	CV^1 (BU)	CV^2 (BU)
Unripe Dominican Republic Dessert Banana Flour (day 2)	63.9 ± 2.5 ($n = 2$)	78.8	225 *95°C (34 min)	211	204	233	218
Unripe Jamaican Dessert Banana Flour (day 1) ($n = 2$)	63.7 ± 1.3	70.4 ± 0.8	216 ± 19.8 *95°C (33.1 ± 0.1 min)	205 ± 21.2	193.5 ± 16.3	210 ± 14.1	191 ± 12.7
Ripe Jamaican Dessert Banana Flour (day 18) ($n = 2$)	59.3 ± 1.2	74.6 ± 0.8	215 ± 35.4 *95°C (31.8 ± 0.1 min)	208.5 ± 33.2	180 ± 28.3	195 ± 28.3	175 ± 21.2
Unripe Dominican Republic Plantain Flour (day 2)	68.2 ± 1.0 ($n = 2$)	77.0	164 *95°C (35 min)	150	161	211	194
Unripe Colombian Plantain Flour (day 1) ($n = 2$)	66.0 ± 2.4	78.9 ± 0.5	172.5 ± 3.5 *95°C (31.9 ± 1.9 min)	166 ± 8.5	152.5 ± 3.5	195 ± 7.1	177.5 ± 3.5
Ripe Colombian Plantain Flour (day 18)	61.4 ± 1.1 ($n = 2$)	78.1	185 *95°C (30 min)	185	135	155	145
Unripe Dominican Republic Cooking Banana Flour (day 2)	58.8 ± 0.4 ($n = 2$)	74.3	170 *95°C (33.4 min)	160	150	185	170

* Temperature (and time) when the peak viscosity was attained

± represents Standard Deviation

Table 6.3.2 Pasting Characteristics of Rapid Viscograms of Starches and Flour from Unripe and Ripe *Musa* Fruits and Reference Starches (See Figures 6.3.1, 6.3.2, 6.3.6 and 6.3.7)

Sample	Starch (%) (d.w.b.)	T^1 (°C)	PV (SNU)	HV^1 (SNU)	HV^2 (SNU)	CV^1 (SNU)	CV^2 (SNU)
Unripe Jamaican Dessert Banana Starch (day 1)	-	79.5	234 *95°C (31.4 min)	229	204	262	274
Ripe Jamaican Dessert Banana Starch (day 18)	-	76.5	259 *95°C (30.4 min)	259	141	184	189
Unripe Colombian Plantain Starch (day 1)	-	79.5	212 *92.7°C	207	147	228	229
Ripe Colombian Plantain Starch (day 18)	-	79.5	251 *86.8°C	233	123	201	205
Unripe Colombian Plantain Flour (day 1)	66.0 ± 2.4 ($n = 2$)	78.01	415 *86.8°C	315	125	214	226
Maize Starch	-	72.1	223 *89.7°C	206	120	260	418
Waxy Maize Starch	-	70.7	272 *75.1°C	100	69	111	114

* Temperature (and time) when the peak viscosity was attained

± represents Standard Deviation

6.4 Conclusions

- The Brabender Viscoamylograph and the Rapid Viscoanalyser demonstrated the pasting behaviour of flours (up to ~68.2% starch) and starches of different *Musa* types. Both techniques relied on similar principles, though the pasting profiles produced by the two instruments were not identical. Therefore, though the pasting profiles between the two techniques were comparable (see Figure 6.3.1), the shapes of the pasting profiles depended on which instrument was used.

- Though the pasting profiles of the flour and starch suspensions of the *Musa* types were similar, there were pasting characteristic differences due to the different swelling behaviour of the starch granules of the different *Musa* types. The pasting characteristics of the *Musa* suspensions were affected by the origin of the fruits, other non-starch components in the flour suspensions, and the degree of maturity of the fruits from which the flours were produced or starches isolated (the ripe starch granules were less stable during cooking than their unripe counterparts).

- There were pasting characteristic differences between the *Musa* starch and flour suspensions which appeared to be *Musa* type dependent. The dessert banana suspensions had higher PVs compared to the plantain and cooking banana suspensions. The unripe cooking banana flour suspension had the least viscous (*i.e.* less stable) retrograded paste compared to the other *Musa* flour suspensions.

- In Chapter 5, it was found that the unripe dessert banana starch granules had a lower AM content than the unripe plantain and unripe cooking banana starch granules, though the unripe dessert banana starch and flour suspensions displayed some of the most viscous (or most stable) pasting profiles. The relationship between the pasting characteristics of the starches and their AM contents was clearly demonstrated by the cereal starches.

However, compared with the cereal starches, the relationship between the pasting characteristics of the *Musa* suspensions and the AM contents of their starch granules was less clear.

- Overall, the results suggest that the molecular characteristics of the AM component and the extent or strengths of the bonding forces within the granule (differences are shown by the different pasting characteristics between the suspensions of the different *Musa* types) are probably the most important determinants of cooking behaviour in *Musa* starch granules. In Chapter 8, the molecular and structural characteristics of the *Musa* starches are discussed in relation to their pasting (cooking) properties.

Chapter 7

Characteristics of the Major Starch Granule-Bound Starch Synthase Protein in *Musa* Starches

7.1 Introduction

In this chapter, preliminary investigations aimed towards finding enzymic differences between different *Musa* types were made on the major starch granule-bound starch synthases (see section 2.4.2.3) in different *Musa* starches. Pea, wheat and potato starches were used as reference samples.

The *Musa* starches were isolated from dessert bananas, plantains, and cooking bananas. The molecular weight of the major starch granule-bound starch synthase (SGBSS) protein of each starch sample was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was used to detect for immunological cross-reactivity between the major SGBSS protein in *Musa* starches with antibodies which had been raised to the SGBSS proteins (*i.e.* GBSSI and GBSSII) in pea embryo starch.

7.1.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Estimation of the molecular weight of a polypeptide is of central importance to the characterisation of proteins (See and Jackowski, 1989). Proteins are more easily separated by zone electrophoresis than by chromatography. Electrophoresis involves the movement of a charged surface (dissolved or suspended material) relative to a stationary liquid by an applied electric field (Shaw, 1969). Polyacrylamide is a stabilising gel media which is used in electrophoresis. The spaces in the polyacrylamide gel network approach macromolecular dimensions so that a molecular sieving effect operates, and the motion of the larger (protein) molecules is preferentially retarded. In electrophoresis, as a result of this molecular sieving and lack of adsorption, an extremely high degree of resolution of the protein molecules can be obtained. The degree of polymerisation of the gel and the gel concentration can be adjusted to optimise the molecular sieving effect for a given separation (Shaw, 1969). Most SDS-polyacrylamide gel electrophoresis is carried out

with a discontinuous buffer system, in which the buffer in the reservoir is of a different pH and ionic strength from the buffer used to cast the gel (Sambrook *et al.*, 1989).

Sodium Dodecyl Sulfate (SDS) binds proteins and gives them a net negative charge and in electrophoresis the negatively charged proteins move towards the positive anode. Under appropriate conditions all reduced polypeptides bind the same amount of SDS on a weight basis (1.4 g SDS/g polypeptide). The amount of SDS bound by a protein is independent of its sequence and is dependent on the structure of the protein (reduced or unreduced), the temperature and the ionic strength of the solution (Sambrook *et al.*, 1989; See and Jackowski, 1989). SDS binds mainly to the hydrophobic regions of polypeptides, whereas the hydrophilic regions bind much less SDS (See and Jackowski, 1989). Separation of SDS-polypeptide complexes by SDS-PAGE is analogous to gel filtration and the separations are dependent on the sizes of the SDS-polypeptide complexes (See and Jackowski, 1989). The electrophoretic mobility is proportional to the molecular weight of a polypeptide or protein only when the charge/mass ratios and the shapes of all the SDS-polypeptide complexes are the same. Therefore, valid molecular weight standards are required for molecular weight determination (See and Jackowski, 1989).

7.1.2 Western Blotting

Western blotting (also known as protein blotting) is a technique for detecting a particular protein by staining with a specific antibody (Stryer, 1988). The Western blotting procedure principally involved the major starch granule-bound protein from a starch sample being applied to denaturing SDS-PAGE which was then electrophoretically transferred to a nitro-cellulose membrane. The membrane was incubated with a first antibody which was directed against the major starch granule-bound protein, and then with second antibodies that were directed against the first antibody. The second antibody

reacted with a developer reagent to produce a colour reaction. This showed the position of the major starch granule-bound protein.

7.2 Materials and Methods

Starch Samples

The starches were isolated from mature green unripe dessert bananas which were grown in the Windward Isles, colour score 1 to 2 (see section 3.2.1), mature green unripe Colombian plantains, and semi-ripe Ugandan cooking bananas. The reference starches comprised pea starch, wheat starch and potato starch.

Antibodies for Western Blotting

The rabbit antibodies, supplied by the John Innes Institute, U.K., comprised:

(i) antibody to the GBSSI (59 kDa) 'waxy' pea embryo starch synthase (1 in 2,000 dilution)

(ii) null serum antibody to the GBSSI (59 kDa) 'waxy' pea embryo starch synthase (control)

(iii) antibody to the GBSSII (77 kDa) pea embryo starch synthase (1 in 5,000 dilution)

(iv) null serum antibody to the GBSSII (77 kDa) pea embryo starch synthase (control)

Equipment and Chemicals

Bio-rad vertical gel electrophoresis apparatus (Bio-rad Laboratories Ltd., Hemel Hempstead, Herts., U.K.) was used. All chemicals were supplied by Sigma Chemical Co., U.K., unless otherwise stated.

Starch Isolation

Starch granules were isolated from the *Musa* fruits, according to section 3.2.2, but instead of air-drying the starch granule-water suspensions, the starch granules were freeze-dried at -40°C for a few hours until dry, using an Edwards high vacuum pump.

7.2.1 Extraction of the Granule-Bound Starch Synthases (Including a Protein Concentrating Step) and SDS-PAGE (see Appendix IIIa for recipes)

Starch granule-bound proteins were isolated by the method of Smith (1990b) with modifications to concentrate the proteins (Dr. A. M. Smith, pers. comm.). Freeze-dried starch granules and reference starches (40-100 mg) were whirlimixed with 800 µl of 2% SDS (cold), then either boiled for 2 min, or not boiled, whirlimixed, and centrifuged for 10 min at 10,000 x g (bench top centrifuge). The supernatant was removed and saved. The pellet which consisted of starch granules which had been stripped of their starch granule-bound proteins was discarded. Acetone (Merck-BDH Co., U.K.) at -20°C was added to the supernatant and the mixture was held in an ethanol dry-ice bath for 30-45 min to precipitate the proteins. After thawing, the samples were centrifuged for 10 min at 10,000 x g (bench top centrifuge). The acetone was then discarded and the 'invisible pellet' freeze-dried for 10 min. The pellet was mixed with 15 µl sample buffer and 5 µl of 17 mM dithiothreitol (DTT). From this, 15 µl was loaded into each well of a 7.5% or 10% polyacrylamide gel. There were eight wells per gel. Molecular weight protein standards, which were used as reference molecular weight markers, comprised 5 µl of Sigma SDS-7, 14-70 kDa narrow range protein markers and 5 µl of Sigma SDS-6H, 30-200 kDa broad range protein markers. The gels were run according to Laemmli (1970). Each gel was run at a constant 200 Volts and took about 45 minutes.

Gels were stained with Coomassie Brilliant Blue R250 for about half an hour with gentle agitation, followed by de-staining overnight with gentle agitation. The positions of the protein bands on the gels were measured and the gels were photographed.

7.2.2 Extraction of the Granule-Bound Starch Synthases (Without the Protein Concentrating Step) and SDS-PAGE (see Appendix IIIb for recipes)

Freeze-dried starch granules (100 mg) were mixed with 800 µl of 2% SDS (cold), then centrifuged for a few minutes at 10,000 x g (bench top centrifuge). The supernatant was discarded together with a pink jelly-like layer on top of the white starch granules. To the lower, white starch granule layer was added 800 µl sample buffer (without bromophenol blue), mixed, boiled for 3 min, mixed again, and centrifuged for 10 min at 10,000 x g (bench top centrifuge). To 40 µl of the sample was added 2 µl of 17 mM dithiothreitol (DTT) and 2 µl of 0.1% bromophenol blue. The suspension was mixed and centrifuged for 3 min at 10,000 x g. 15 µl of the supernatant was loaded into each well of a 7.5% polyacrylamide gel. There were eight wells per gel. Molecular weight protein standards, which were used as reference molecular weight markers, comprised 5 µl of Sigma SDS-7, 14-70 kDa narrow range protein markers and 5 µl of Sigma SDS-6H, 30-200 kDa broad range protein markers. The gels were run according to Laemmli (1970). Each gel was run at a constant 200 Volts and took about 45 minutes.

Gels were stained with Coomassie Brilliant Blue R250 for about half an hour with gentle agitation, followed by de-staining overnight with gentle agitation. The positions of the protein bands on the gels were measured and the gels were photographed.

7.2.3 Preparation for Western Blotting

The sample preparation and gel electrophoresis procedure prior to Western blotting was as described in section 7.2.2, but without the inclusion of molecular weight protein

standards, or Bromophenol Blue in the SDS-PAGE sample preparation, or staining and de-staining procedures after SDS-PAGE. The SDS-polyacrylamide gels were soaked in Western blotting buffer (see Appendix IIIb). After soaking filter paper and NC-100 nitrocellulose pieces in blotting buffer the SDS-polyacrylamide gels were placed onto the filter paper and the pieces of nitrocellulose were placed on top. The Western blot was kept cool and run overnight at 30 Volts and 0.15 Amps.

7.2.4 Western Blotting Procedure

Blots were blocked for two hours in phosphate buffered saline (PBS), 3% bovine serum albumin (BSA), and 2% dried milk powder. The first antibody, in PBS and 3% BSA was added and the blot was incubated for 1-2 h. The blots were washed for 2 x 5 min in PBS, 0.1% Tween, and then 2 x 5 min in PBS, 1M NaCl, 0.5% Tween. They were then blocked for 15 min in PBS and 3% BSA, followed by an incubation for 1-2 h with 1/1000 or 1/1500 Sigma alkaline phosphatase-conjugated goat anti-rabbit antibody (7.5 µl Sigma immunochemicals A-8025 anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate). The blots were then carried through a series of washing steps: 2 x 5 min in PBS, 0.1% Tween; 1 x 10 min in PBS, 1M NaCl, 0.5% Tween; 3 x 5 min in PBS, 0.1% Tween; 1 x 5 min in PBS; and washed briefly in distilled water. All of the steps were carried out with gentle agitation. Sufficient developer was added to cover the surface of the Western blots and prevent drying. The developer used was Western blue stabilised substrate for alkaline phosphatase (Promega, Southampton, U.K.). The developing step was stopped by immersing the blot in an excess of water. The blots were dried between M & M Whatman filter paper (Whatman International Ltd., U.K.) and photographed.

7.3 Results and Discussion

GBSS Extraction

The methods, described in section 7.2.1 and 7.2.2, were two slightly different procedures which were used to extract the major starch granule-bound starch synthase proteins from the starches. In section 7.2.2, as a preliminary 'cleaning' step, starch granules were mixed with 2% SDS (cold) and centrifuged. Then, to the pellet of starch granules was added sample buffer and DTT, which was then mixed, boiled, and centrifuged. The supernatant was loaded into wells of the gel. In section 7.2.1, however, the 'cleaning' step was not included and the supernatant obtained after the first centrifugation step (and not the second, as in section 7.2.2) was used for the polyacrylamide gels.

Calibration of the Gels Using Molecular Weight Protein Markers

The calibration plots, constructed from the molecular weight protein markers, were both linear (Figure 7.3.1, $r = 0.989$ and 0.977).

SDS-Polyacrylamide Gels

The SDS-polyacrylamide gels of the starch samples of the different *Musa* types and reference starches are shown in Plates 7.3.1, 7.3.2 and 7.3.3. Plate 7.3.3 represents a typical SDS-polyacrylamide gel which was used to produce the Western blots (molecular weight protein markers were not included when preparing the polyacrylamide gels which were later used for Western blotting).

Molecular Weight of the Major Protein Band

On all of the SDS-polyacrylamide gels (Plates 7.3.1 to 7.3.3), there was a recurring simple pattern - a single major protein band. The major starch granule-bound starch synthase protein (shown by the major protein band) of the plantain, cooking banana and dessert banana *Musa* starches and the reference starches was approximately 59 kDa in molecular

weight. Similarly, the GBSSI protein in developing pea embryo starch also has a molecular weight of 59 kDa (*e.g.* see Smith and Denyer, 1992).

Higher Concentrations of Protein were seen in the Boiled Samples on the Gels (Probably due to Starch Granule Swelling)

The protein concentrating procedure (section 7.2.1) increased the number of bands on the gels - there were many other protein bands both higher and lower in molecular weight than 59 kDa (especially seen in Plate 7.3.1). The starch samples of the different *Musa* types all displayed the major protein band, both with and without the boiling step (Plates 7.3.1 and 7.3.2, though not clear in the latter photograph).

As seen in Plate 7.3.1, the boiled starch samples generally had higher concentrations of the major SGBSS protein (59 kDa) compared with the non-boiled starch samples. This observation suggests that boiling in 2% SDS 'lifted-off' more of the major SGBSS protein from the surfaces of the starch granules. The starch granules swelled considerably in aqueous solution, and hydration in cold 2% SDS alone may have 'pushed out' the superficial and loosely attached starch synthase proteins. Boiling may have caused an increase in water absorption and swelling power of the starch granules resulting in previously 'inaccessible' starch synthases being released from within the granules. These results in Plate 7.3.1 were consistent with the view that the SGBSS proteins become embedded within the 'latticework' of the growing starch polymer (and therefore the SGBSS proteins become more inaccessible to substrate and inactive with growth of the starch granule) (see Figure 2.4.2).

It is possible that the starches used in the SDS-polyacrylamide gel electrophoresis experiments had different molecular structures which would probably result in different swelling capacities. For example, the swelling pattern of banana starch has been reported

as being intermediate between that of wheat starch and potato starch (French, 1984). Thus, due to the different swelling capacities of the different starches used in the investigations, it is likely that the different *Musa* starches and the reference starches would have released comparatively different amounts of protein in aqueous solution. Thus, the amounts (or depths of colour due to staining) of SGBSS protein (shown as bands on the SDS-PAGE gels) for each starch sample did **not** reflect the total content of SGBSS protein present in the starch, *i.e.* the technique was not quantitative.

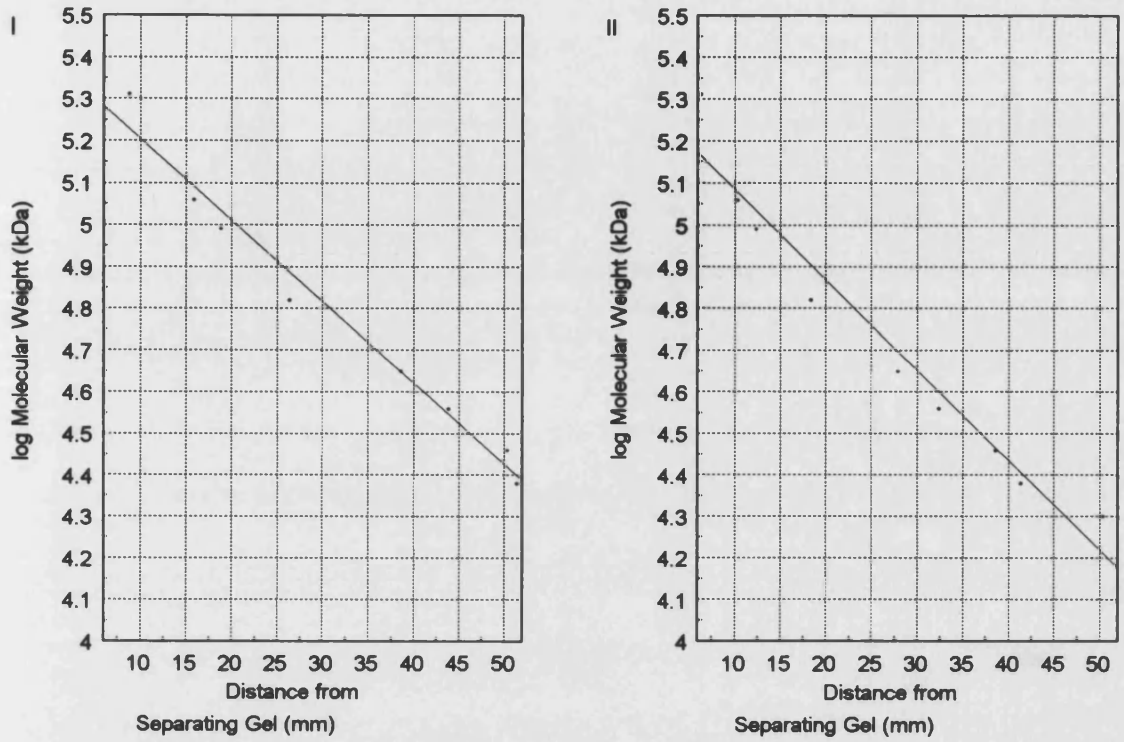
Other Minor Protein Bands on the Gels

In Plate 7.3.1, other protein bands were visible besides the major GBSSI protein band, especially when a higher concentration of the dessert banana starch was used in the SGBSS extraction procedure. In Plates 7.3.2 and 7.3.3, these 'extra' protein bands were not as clear as in Plate 7.3.1, as lower concentrations of starch were used in the former two gels.

In Plates 7.3.2 and 7.3.3, a protein which was lower in molecular weight than the major SGBSS protein (59 kDa) was seen in all of the *Musa* starch samples and the reference starch samples (just below the major protein band), though this protein band was barely visible in the photograph.

In Plate 7.3.3, a protein band of approximately 77 kDa was observed on the SDS-polyacrylamide gel for the pea starch sample (as expected), though this band was barely visible in the photograph of the gel.

Figure 7.3.1 SDS-PAGE Molecular Weight Calibration Plots: I, For the 7.5% Gel (Plate 7.3.1)**, and II, For the 10% Gel** (Plate 7.3.2)



The molecular weight protein standards comprised 205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa and 29 kDa (30-200 kDa broad range protein markers, Sigma SDS-6H), and 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa and 14.2 kDa (14-70 kDa narrow range protein markers, Sigma SDS-7).

The linear regression correlations for the above plots I and II, were $r = 0.989$ and $r = 0.977$, respectively.

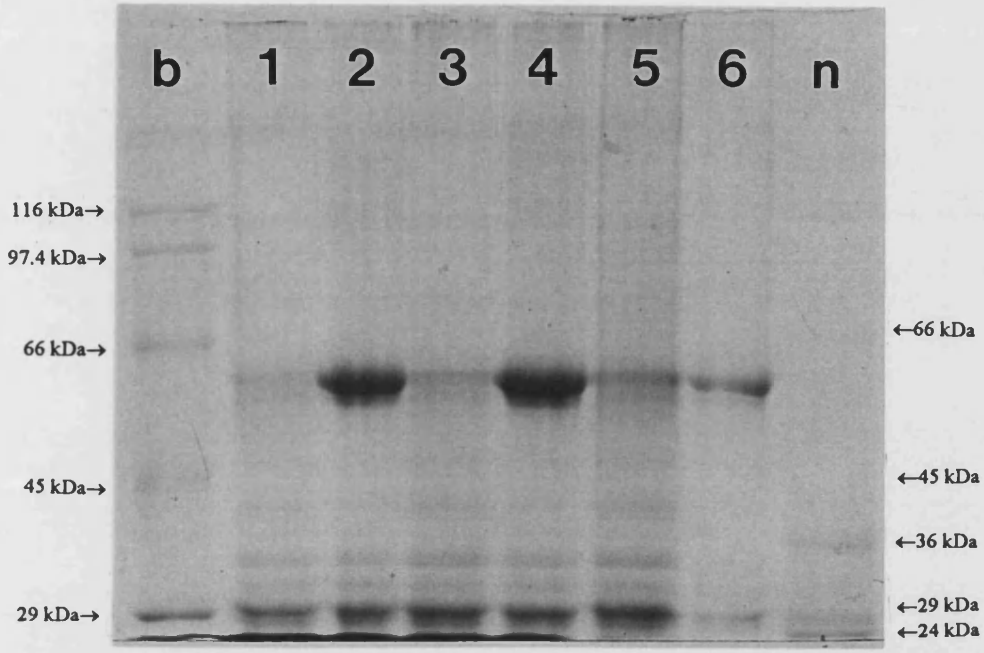


Plate 7.3.1 Photograph of a 7.5% SDS-Polyacrylamide Gel of the Major Starch Granule-Bound Proteins of Unripe Dessert Banana Starch

The SGBSS proteins were extracted according to section 7.2.1.

Key:-

b, Sigma SDS-6H, 30-200 kDa broad range protein markers,

1, 40 mg (not boiled),

2, 40 mg (boiled),

3, 50 mg (not boiled),

4, 50 mg (boiled),

5, 60 mg (not boiled),

6, 60 mg (boiled),

n, Sigma SDS-7, 14-90 kDa narrow range protein markers.

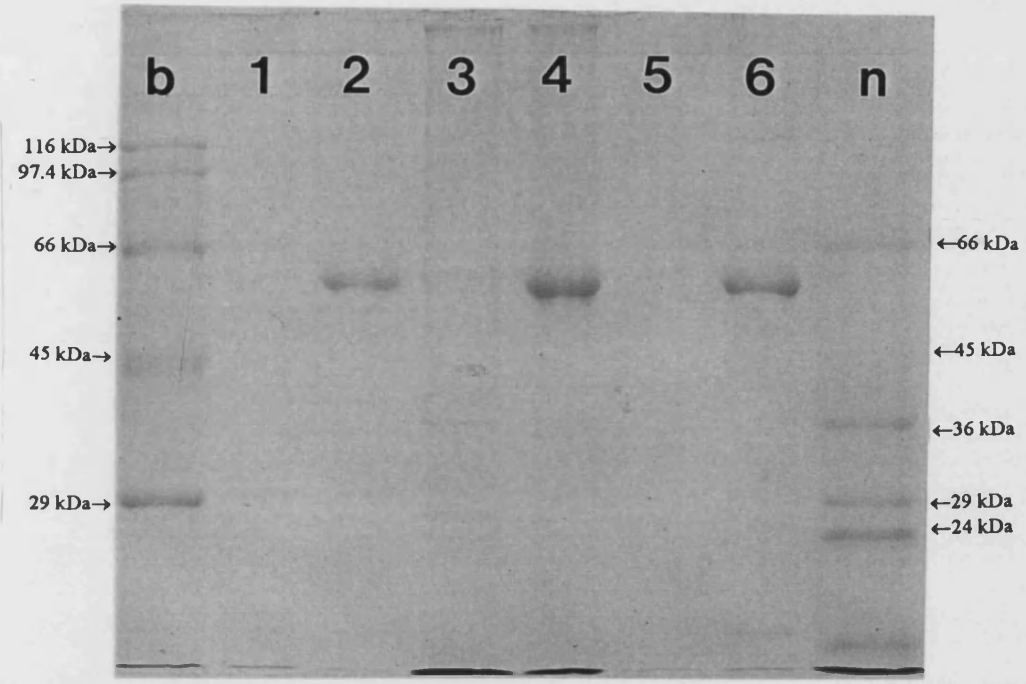


Plate 7.3.2 Photograph of a 10% SDS-Polyacrylamide Gel of the Major Starch Granule-Bound Proteins of Unripe Dessert Banana Starch, Unripe Plantain Starch, and Semi-Ripe Cooking Banana Starch

The SGBSS proteins were extracted according to section 7.2.1.

Key:-

b, Sigma SDS-6H, 30-200 kDa broad range protein markers,

1, Unripe dessert banana starch (50 mg, not boiled),

2, Unripe dessert banana starch (50 mg, boiled),

3, Semi-ripe cooking banana starch (50 mg, not boiled),

4, Semi-ripe cooking banana starch (50 mg, boiled),

5, Unripe plantain starch (50 mg, not boiled),

6, Unripe plantain starch (50 mg, boiled),

n, Sigma SDS-7, 14-70 kDa narrow range protein markers.

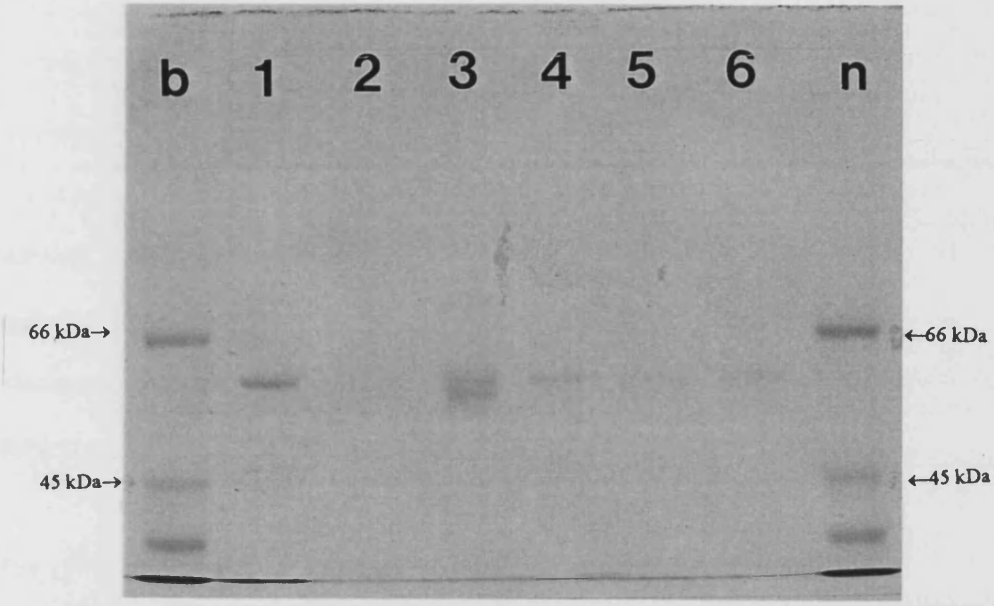


Plate 7.3.3 Photograph of a 7.5% SDS-Polyacrylamide Gel of the Major Starch Granule-Bound Proteins of Pea Starch, Wheat Starch, Potato Starch, and Starches from Unripe Dessert Bananas and Unripe Plantains

The SGBSS proteins were extracted according to section 7.2.2.

Key:-

b, Sigma SDS-6H, 30-200 kDa broad range protein markers,

1, Unripe dessert banana starch, sample (i),

2, Unripe dessert banana starch, sample (ii),

3, Unripe plantain starch,

4, Pea starch,

5, Wheat starch,

6, Potato starch,

n, Sigma SDS-7, 14-90 kDa narrow range protein markers.

Immunological Cross-Reactivity Between the SGBSS proteins in the *Musa* Starches with Antibodies Raised to the GBSSI and GBSSII Enzymes of Pea Embryo Starch

Western blotting was used to detect similarities between the SGBSS proteins of dessert banana, plantain, wheat and potato starches, with those of pea starch. The antibodies which were used had been raised to the 'waxy' GBSSI (59 kDa) protein and also the GBSSII (77 kDa) protein of developing pea embryo starch. The results of the Western blotting experiments are shown in Plates 7.3.4 and 7.3.5. The SGBSS proteins were transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane by electro-blotting. The Western blotting procedure involved coating the NC-100 nitrocellulose with milk and BSA globulins, which blocked the nitrocellulose membrane around the proteins which were stuck onto it. The protein bands were developed by incubation with the antibody followed by alkaline-phosphatase-conjugated goat anti-rabbit antiserum.

The pre-immune or null serum reactions (controls) showed no bands (Western blots not shown).

Lane 2, in Plates 7.3.3, 7.3.4 and 7.3.5

Lane 2, in Plates 7.3.3, 7.3.4 and 7.3.5, showed conflicting results to those of the same *Musa* type in lane 1. The results of lane 2 were not consistent with other gels which were run (results not shown). Therefore, the results of lane 2 were believed to be due to experimental error. The gels in Plates 7.3.3, 7.3.4 and 7.3.5 were shown despite the confusing results of lane 2, to show the results of the starches from the other different plant species.

The Antibodies Raised to the GBSSI and GBSSII Proteins of Pea Embryo Starch Reacted Specifically with the Major GBSSI Protein in the *Musa*, Pea, Wheat and Potato Starches

The Western blot in Plate 7.3.4 shows that the antibody raised to the GBSSI (59 kDa) protein of pea embryo starch reacted specifically with the major SGBSS protein in the dessert banana, plantain, pea, wheat and potato starches. This result indicates that there are similarities in amino acid sequences of this starch biosynthetic protein between the different *Musa* types and different plant species.

Antibodies which had been raised to the GBSSII protein in pea embryo starch were used to create the Western blot shown in Plate 7.3.5. The results of this blot show that there was considerable immunological cross reactivity between the antibodies raised to the GBSSII enzyme and the major SGBSS protein (59 kDa). This is not surprising, as Dry *et al.* (1992) reported that the GBSSI and GBSSII proteins in developing pea embryo starch have similar amino acid sequences (section 2.4.2.3).

On the Western blot, in Plate 7.3.5, a protein band of around 77 kDa was clearly visible in the plantain starch sample. This suggested that the SGBSS proteins in plantain starch had immunologically cross-reacted with the antibody raised to the GBSSII protein of pea embryo starch, *i.e.* there were amino acid sequence similarities between the GBSSII starch biosynthetic proteins in plantain starch and pea starch. As expected, pea starch displayed a protein band with a molecular weight of around 77 kDa (though this band is not very clear on the photograph). As this protein band was very faint, this suggests that there was only a very small amount of this GBSSII protein to cross-react with the antibody. The wheat starch and potato starch samples did not appear to have this 77 kDa protein, which suggested that either they did not have this protein, or this protein was not concentrated

enough to stain, but may have done so over a longer period of time with the developer reagent.

The results of both Western blots clearly indicated that the major SGBSS protein in different *Musa* types was similar in amino acid sequence to the same starch biosynthetic protein in other plant species.

Minor Protein Bands in the Western Blots

For most of the starch samples in Plates 7.3.4 and 7.3.5, a protein band slightly lighter than the 59 kDa protein can be seen. This protein band is also seen in Plates 7.3.1, 7.3.2 and 7.3.3 (mentioned previously). The immunological cross-reactivity between this protein and either of the antibodies raised to the GBSSI or GBSSII proteins suggests that this protein might also be a starch biosynthetic enzyme, or be connected with starch biosynthesis in some way.

In Plates 7.3.4 and 7.3.5, there were slight differences between the banding patterns of the plantain and dessert banana starch samples. For instance, the plantain starch sample had two protein bands below the major GBSSI band, which must have had amino acid sequence similarity to the pea embryo starch GBSSI (59 kDa) protein (to which the antibody was raised) (Plate 7.3.4). In Plate 7.3.5, the dessert banana starch and the plantain starch samples both appeared to have additional proteins that cross-reacted with the GBSSII antibody: the dessert banana starch sample showed a protein band of approximately 95 kDa (lane 1); and the plantain starch sample showed a protein band of around 77 kDa, a protein band of between 59 kDa and 77 kDa, and a protein band which was lower in molecular weight than the low molecular weight 'double' band as seen in Plate 7.3.4 (mentioned above). Therefore, the proteins which were visible in Plate 7.3.5,

but not in Plate 7.3.4, were possibly similar in amino acid sequence with the GBSSII (77 kDa) protein, but not with the GBSSI (59 kDa) protein, and *vice versa.*

The presence of a protein band in one starch sample and not in another did not necessarily mean that the starch sample not displaying this protein band did not have this particular protein. For instance, the extraction procedure may not have removed a sufficient amount of this other 'minor' protein in all of the starch samples (perhaps as a result of their different structures, compositions, and therefore different swelling capacities), or the concentration of this protein in the starch sample was too low to stain with the developer reagent.

The minor protein bands on the SDS-polyacrylamide gels may also have been starch biosynthetic enzymes, though this study was not taken far enough to discount the possibility that they were contaminants. For example, the lower molecular weight proteins seen in all of the starch samples (Plates 7.3.1, 7.3.2 and 7.3.3) may have been contaminants from the pink debris earlier on in the starch granule-bound protein extraction procedure, which was probably due to protein flocculated by anthocyanins or other phenolic compounds.

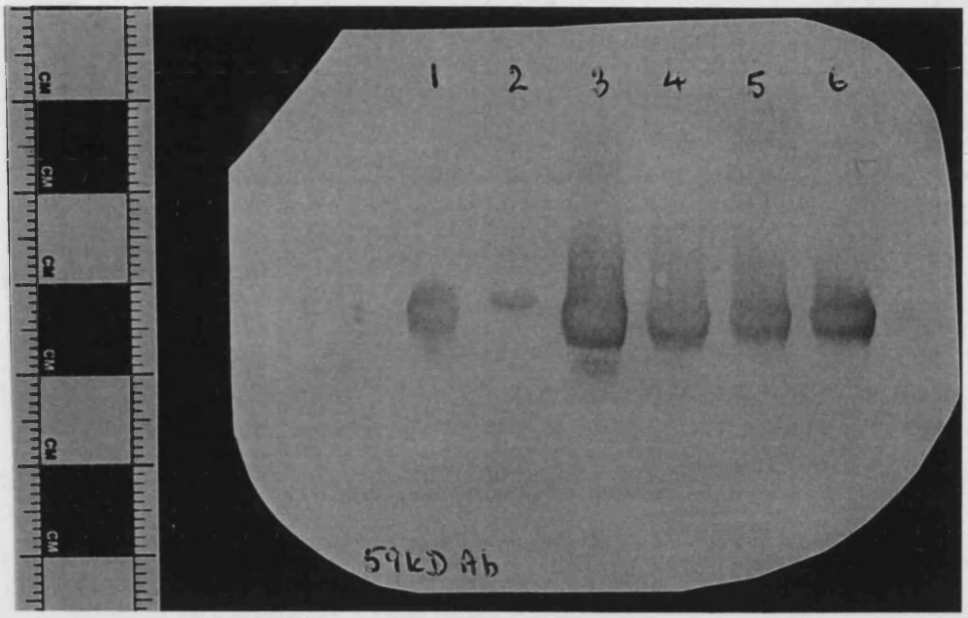


Plate 7.3.4 Photograph of Western Blot of 7.5% SDS-Polyacrylamide Gel Using Antibodies to the 59 kDa Pea Embryo Starch Synthase Protein (GBSSI)

Key:-

1, Unripe dessert banana starch granules, sample (i),

2, Unripe dessert banana starch granules, sample (ii),

3, Unripe plantain starch granules,

4, Pea embryo starch granules,

5, Wheat starch granules,

6, Potato starch granules.

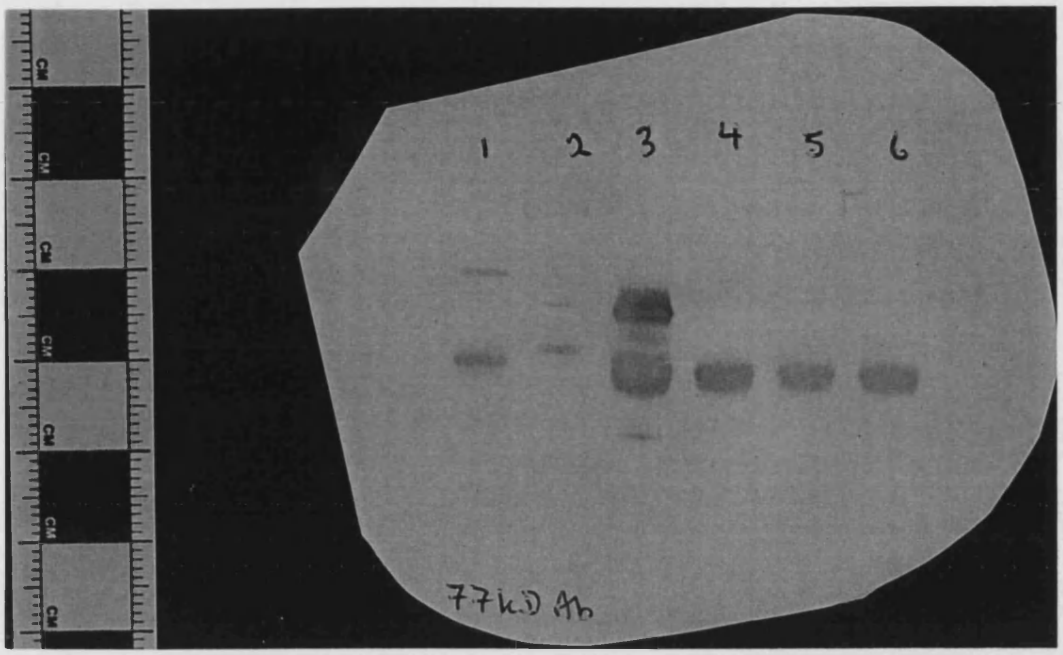


Plate 7.3.5 Photograph of Western Blot of 7.5% SDS-Polyacrylamide Gel Using Antibodies to the 77 kDa Pea Embryo Starch Synthase Protein (GBSSII)

Key:-

1, Unripe dessert banana starch granules, sample (i),

2, Unripe dessert banana starch granules, sample (ii),

3, Unripe plantain starch granules,

4, Pea embryo starch granules,

5, Wheat starch granules,

6, Potato starch granules.

7.4 Conclusions

- The boiling step removed more of the major SGBSS protein from the starch granules.

- The major SGBSS protein in plantain starch, cooking banana starch and dessert banana starch was similar in molecular weight (approximately 59 kDa). This protein was also similar in molecular weight to the major SGBSS protein in pea starch, wheat starch and potato starch.

- The major SGBSS protein in plantain starch and dessert banana starch was found react specifically with antibodies raised to the major SGBSS protein (*i.e.* GBSSI) of pea embryo starch.

- Overall, the results suggested similarities in the major SGBSS protein between the different *Musa* types and different plant species.

Chapter 8

General Discussion and Conclusions

8.1 Introduction

The experiments described in this thesis were aimed towards characterising and comparing the physical, molecular and structural properties of starches from different *Musa* types in relation to their cooking behaviour. A better understanding of the starch granule characteristics, the molecular and structural characteristics of the major starch polymers of which the starch granules are composed (*i.e.* AM and AP), and the functional properties of starch granules in bananas and plantains, would be particularly beneficial to *Musa* breeders, whose ultimate aim is to produce disease resistant hybrids which retain the quality characteristics of the consumer acceptable fruits. The following summarises the results and the conclusions of the experiments. Suggestions have been made for future research using other techniques.

8.2.1 Major Starch Granule-Bound Starch Synthase (SGBSS)

During starch biosynthesis, AM is produced in the starch granule under the genetic instruction of the granule-bound starch synthase (GBSS) protein (also known as the *Waxy*, or *Wx* protein). Preliminary experiments were done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to detect for differences (or similarities) in this major granule-bound starch synthase protein between the starches of different *Musa* types. The major GBSS protein in plantain, dessert banana and cooking banana starches was similar in molecular weight (~59 kDa). This major *Musa* starch protein also showed molecular weight and amino acid sequence similarities (as shown by immunological cross-reactivity) to the major GBSS protein in pea starch, wheat starch and potato starch. Hence, the major GBSS protein appeared to be a conserved enzyme and there did not appear to be any obvious differences in the characteristics of this protein either between different *Musa* types or between different plant species.

Since the major SGBSS protein (*i.e.* GBSSI), which is responsible for the production of AM, was found to have similar characteristics both within and between different plant species, and if the underlying cause for the differences in the pasting characteristics between different *Musa* types is found to lie in the AM component, it is possible that distinguishable *Musa* type differences could lie in other factors associated with this SGBSS protein. For example, the level of expression, or rate of activity, or inhibitory effects upon this starch biosynthetic enzyme (*e.g.* by other proteins), which could be more active in one *Musa* type or cultivar than in another, might be responsible for physical and functional property differences between starches. It is also possible that other enzymes of starch biosynthesis, such as the Branching Enzymes (BEs) and their enzymic regulation, could be important for differences in the starch characteristics and the functional properties of the starch granules of different *Musa* types. Or perhaps the association or relationship between the GBSS, SSS and BEs is an important distinguishable feature between different *Musa* types. It is clear that much more research is needed in the areas of starch biosynthesis in *Musa* fruits.

8.2.2 Amylose Contents of the *Musa* Starches

Though the major GBSSI protein in the starches of the different *Musa* types appeared to be similar, minor differences were observed between the different *Musa* starches in their AM contents, determined by GPC. The AM contents of the plantain starches were similar to that of the cooking banana starch (they ranged from 21.6-23.9%). The dessert banana starches had even lower AM contents (16.8-17.9%).

8.2.3 Molecular Characteristics of the *Musa* Starches

Overall, the results of the GPC experiments did not suggest any major differences between the molecular characteristics of the AP components in the starches of the different *Musa* types.

8.2.4 Structural Characteristics of the *Musa* Starches

Studies using powder X-ray diffraction demonstrated that starches from different plant species displayed different crystalline patterns, though the structural arrangement of the polymers within the starches of the different *Musa* types was the same; dessert banana starch and plantain starch both displayed a C-type structure, which is a transitional type between the A-type structure (in waxy maize starch granules) and the B-type structure (in potato starch granules).

The degree of starch crystalline order (*i.e.* crystallinity) of the unripe plantain starch and the unripe and ripe dessert banana starches were similar. The percentage of starch crystallinity was higher in the ripe compared to the unripe *Musa* fruits due to enzymic degradation and re-ordering of the starch polymers.

8.2.5 Physical Appearances and Sizes of the *Musa* Starch Granules

In general, the physical appearances of the starch granules of the different *Musa* types were similar in size and shape. The *Musa* starch granules ranged from 18.9-22.1 μm in diameter. The shapes of the *Musa* starch granules were oyster-shell and irregular (similar to those of potato starch granules). The particle sizes decreased with increasing ripeness of the fruits from which the starches were isolated due to enzymic degradation of the starch granules. As the differences between the particle sizes of the different *Musa* starches were so small, it would be unlikely that any significant differences in the functional properties of these starches could be due to their particle sizes. This was found to be the case in the pasting experiments; there did not appear to be any obvious relationship between these physical characteristics and the cooking behaviour of the *Musa* starch granules. Similarly, Goering (1978) proposed that the pasting properties of starches were probably due to granule structure and not particle size.

8.2.6 Starch Contents of the *Musa* Flours

Starch was the major component of the *Musa* flours (up to ~68.2%). The starch contents of the *Musa* flours decreased with increasing ripeness of the fruits from which the flours were made (this was also seen in Appendix Ic).

8.2.7 Pasting Characteristics

The pasting characteristics of the *Musa* flour and starch suspensions were very much dependent on the technique used to study them. The pasting properties of the *Musa* flour and starch suspensions of the same *Musa* type showed differences due to the non-starch components in the flour suspension. The degree of maturity and the country of origin of the fruits also affected the pasting profiles. The pasting profiles of the different *Musa* types were similar, but they possessed different pasting properties due to the different swelling behaviour of the starch granules. The different swelling behaviour of the *Musa* starch suspensions suggested possible differences in the molecular association of the starch polymers (such as the strength or extent of inter- or intra-molecular hydrogen-bonding) within the starch granules of the different *Musa* types.

8.2.7.1 Relationship Between the Molecular and Structural Characteristics of the *Musa* Starches and their Pasting Characteristics

The AM contents of the *Musa* and cereal starches, determined using the GPC technique, were considered in relation to their pasting characteristics. There was a consistent relationship between the pasting characteristics of the cereal starches and their AM contents throughout the entire pasting process, as is reported in the literature. However, the same relationship was less clear for the *Musa* starches. Though the pasting characteristics of the *Musa* starches could be related to their AM contents during retrogradation, this relationship was not entirely consistent for all of the *Musa* starches. Thus, the relationship between the pasting characteristics of the *Musa* starches and their

AM contents was generally inconclusive. The results of the pasting experiments suggested possible differences in the molecular characteristics and the association of the polymer components in the starch granules of the different *Musa* types. Differences in the molecular characteristics of the AP component in the starch granules of the different *Musa* starches, as determined by GPC, were unclear. Therefore, it is possible that differences between *Musa* types might be found in the molecular characteristics of the AM component of the starch granules. In the literature, the molecular size and fine structure of the AM component has also been suggested as a possible factor controlling the swelling of starch granules and the peak viscosity during cooking, as a result of its association with the crystalline AP component (Kayisu *et al.*, 1981; Banks *et al.*, 1974; Biliaderis *et al.*, 1981; Greenwood, 1979).

The structural characteristics of the starch granules (the crystalline arrangement of the starch polymers and the starch crystallinity) of the dessert banana and plantain *Musa* types, determined by powder X-ray diffraction, were similar as were the pasting profiles of the *Musa* flour and starch suspensions. In contrast, as well as having a much lower AM content than the *Musa* starches, the waxy maize starch granules displayed both a different type of crystalline pattern and a dramatically different pasting profile to those of the *Musa* types.

8.3 Conclusions

The *Musa* fruits used in the experiments were grown in different countries, and it was not known to what extent, if any, the differences between the results of the experiments were due to varietal, environmental, or agronomic factors. Temperature and environmental conditions have been reported to contribute to property differences between starches (cited by Shannon and Garwood, 1984; Asaoka *et al.*, 1984). Despite this, the results from the experiments suggest that the starch granules of the different *Musa* types possess

similar physical and structural characteristics. The molecular characteristics of the AP component in the starch granules of the different *Musa* types also appeared to be similar. From the results, it seems likely that the pasting properties of *Musa* types may be primarily determined by the fine molecular characteristics of the starch polymers, particularly the AM component (and its molecular association) within the starch granules. More information about the fine molecular characteristics of the AM and AP components is required, using a more sensitive technique than GPC, in order to determine whether there are any distinguishable molecular differences between the starches of different *Musa* types which cause them to paste in different ways. For example, nuclear magnetic resonance (n.m.r.) spectroscopy (see below) could be used to determine the degree of branching of AM; and high-performance size-exclusion chromatography (HPSEC) could be used to investigate various molecular characteristics of the AM component (*i.e.* polydispersibility, degree of branching, molecular size, *etc*).

8.4 Proposals for Future Research

In the following sections, suggestions have been made for future experimental research on *Musa* fruits.

8.4.1 Degree of Fruit Maturity

The exact ages of the fruits post-harvest, and the effects of storage, transportation and handling treatments on the fruits, may have been factors which contributed to some of the fruit quality characteristic differences between the *Musa* types. In the experiments described in this thesis, the degree of ripeness of the *Musa* fruits was assessed subjectively using a peel colour score index chart, by measuring the pulp to peel ratio and the starch contents of the *Musa* flours, and by estimating the ages of the fruits from the day when they were harvested to the day when they were purchased. Clearly, the best experimentation on unripe *Musa* fruits should be carried out in the field, or on fruits

grown by the researcher. However, in future research, it might be useful to use more indices of fruit maturity when comparing *Musa* types and cultivars. For example, the total soluble solids content (% Brix) of the fruit can be a useful index of maturity, as demonstrated by Blankenship *et al.* (1993), since the amount of sugar in the fruits usually increases as the fruit matures and ripens.

8.4.2 HPSEC

The differences in the pasting characteristics between the *Musa* starches could be the result of subtle differences in the molecular characteristics (*e.g.* chain lengths, degree of branching) of their AM or AP components, or both. The technique of GPC was used primarily to examine the molecular characteristics of the AP component of the starches. However, distinguishable molecular differences in the AP components between the different *Musa* types were not found. GPC is a relatively slow technique with limitations of relatively slow flow rates and pressures. Also, the chemical analysis of the GPC eluted fractions is time consuming, uses hazardous chemicals, and could be an area subject to error arising from contamination (cited by Ong *et al.*, 1994). Thus, it would be preferable to use a faster, more sensitive technique, without the need for chemical analysis of the eluted fractions. High-performance size-exclusion chromatography (HPSEC) is a relatively new development of the GPC technique (though it is a much more expensive technique than GPC). HPSEC is characterised by high pressures, sophisticated equipment, and short time separations made possible by small, porous-particles that are packed into highly efficient columns which improve the resolution (Yau *et al.*, 1979; Kobayashi *et al.*, 1985). The results of the experiments described in this thesis indicate that there is a need to accurately define the molecular characteristics of the AM **and** AP polymer components of the starch granules of different *Musa* types using more sensitive techniques than GPC. HPSEC could provide more useful information on the molecular characteristics of both of the polymer components of the starch granule, *e.g.* HPSEC

could provide data to determine the polydispersibility of each polysaccharide fraction (*i.e.* the distribution of molecular weights). Hizukuri (1986) used a molecular weight detector (a low-angle laser light scattering detector) to determine the lower molecular weights of the debranched AP components and revealed tetramodal distribution profiles of potato AP. In contrast, when the GPC technique was used in Chapter 5, a bimodal elution profile was observed for potato starch and all of the *Musa* starches (Figure 5.2.2). From the results of the pasting experiments, it is proposed that the analysis of the chain length and the degree of branching of particularly the AM component might help to explain why the different *Musa* types behave differently during cooking and cooling.

8.4.3 N.m.r Spectroscopy

Another technique which could provide more structural information on the starch polymers and possibly reveal any differences between *Musa* types is nuclear magnetic resonance (n.m.r.) spectroscopy. N.m.r. spectroscopy is a useful non-destructive technique for the determination of oligosaccharide structures, and is much less complex and time consuming than methods such as GPC. ^{1}H-n.m.r. spectroscopy can be used to determine the degree of branching of starch-derived polysaccharides, *e.g.* amyloses (Gidley, 1985).

8.4.4 DSC

Differential Scanning Calorimetry (DSC) has been found to be the preferred means of studying the gelatinisation of starch as compared to other more conventional techniques such as the polarised optical light microscope (Atwell *et al.*, 1988). DSC is the most commonly used technique in starch gelatinisation and reveals an endothermic event (often equated with the thermal melting of crystallites) at temperatures similar to those at which structural changes are observed. DSC could be used to look for differences in the AM contents (*e.g.* Sievert and Holm, 1993; Kugimya and Donovan, 1981) and the enthalpies

of gelatinisation between different *Musa* types and cultivars. This information, used in conjunction with that obtained from HPSEC experiments using *Musa* starches, could provide a better understanding of the molecular and structural properties of the starch granules of different *Musa* types, in relation to their functional behaviour.

Bibliography

Aked, J. and McDowell, I. (1993) An investigation of astringency factors in the pulp of green bananas. Natural Resources Institute report. Project No. 10063.

Asaoka, M., Blanshard, J. M. V. and Rickard, J. E. (1991) Seasonal effects on the physico-chemical properties of starch from four cultivars of cassava. *Starch/Stärke* **43**, 455-459.

Asaoka, M., Blanshard, J. M. V. and Rickard, J. E. (1992) Effects of cultivar and growth season on the gelatinisation properties of cassava (*Manihot esculenta*) starch. *Journal of the Science of Food and Agriculture* **59**, 53-58.

Asaoka, M., Okuno, K., Sugimoto, Y. Kawakami, J. and Fuwa, H. (1984) Effect of environmental temperature during development of rice plants on some properties of endosperm starch. *Starch/Stärke* **36**, 189-193.

Atwell, W. A., Hood, L. F., Lineback, D. R., Varriano-Marston, E. and Zobel, H. F. (1988) The terminology and methodology associated with basic starch phenomena. *Cereal Foods World* **33**, 306-311.

Bamidele, E. A., Cardoso, A. O. and Olaofe, O. (1990) Rheology and baking potential of wheat/plantain composite flour. *Journal of the Science of Food and Agriculture* **51**, 421-424.

Banks, W. and Greenwood, C. T. (1975) *Starch and its components*. Edinburgh University Press, Edinburgh, U.K.

Banks, W., Greenwood, C. T. and Muir, D. D. (1974) Studies on starches of high amylose content. Part 17. A review of concepts. *Starch/Stärke* **26**, 289-300.

Berríos, M. L. and González, M. A. (1971) Extraction, purification and amylose content of some starches. *Journal of Agriculture of the University of Puerto Rico* **55**, 263-264.

Biliaderis, C. G., Grant, D. R. and Vose, J. R. (1981) Structural characterisation of legume starches. I. Studies on amylose, amylopectin and beta-limit dextrins. *Cereal Chemistry* **58**, 496-502.

Blankenship, S. M., Ellesworth, D. D. and Powell, R. L. (1993) A ripening index for banana fruit based on starch content. *Horticultural Technology* **3**, 338-339.

Blanshard, J. M. V. (1986) The significance of the structure and function of the starch granule in baked products. In: *Chemistry and Physics of Baking*. Ed. Blanshard, J. M. V., Frazier, P. J. and Galliard, T. 1-13. Royal Society of Chemistry, U.K.

Blanshard, J. M. V. (1987) Starch granule structure and function: a physicochemical approach. In: *Starch: Properties and potential*. Ed. Galliard, T. 16-54. John Wiley and Sons, U.K.

Blanshard, J. M. V., Bates, D. R., Muhr, A., Worcester, D. L. and Higgins, J. S. (1984) Small-angle neutron scattering studies of starch granule structure. *Carbohydrate Polymers* **4**, 427-442.

Bradbury, A. G. W. and Bello, A. B. (1993) Determination of molecular size distribution of starch and debranched starch by a single procedure using high-performance size-exclusion chromatography. *Cereal Chemistry* **70**, 543-547.

Caesar, G. V. (1932) Consistency changes in starch pastes. *Industrial and Engineering Chemistry* **24**, 1432-1435.

Chinachoti, P. and Steinberg, M. P. (1986) Crystallinity of waxy maize starch as influenced by ambient temperature absorption and desorption, sucrose content and water activity. *Journal of Food Science* **51**, 997-1000, 1036.

Collison, R. (1968a) Starch retrogradation. In: *Starch and its derivatives*. 4th edition. Ed. Radley, J. A. 194-202. Chapman and Hall Ltd., London, U.K.

Collison, R. (1968b) Swelling and gelation of starch. In: *Starch and its derivatives*. 4th edition. Ed. Radley, J. A. 168-193. Chapman and Hall Ltd., London, U.K.

Cooke, D. and Gidley, M. J. (1992) Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition. *Carbohydrate Research* **227**, 103-112.

Dadzie, B. K. (1995) Post-harvest criteria and methods for routine screening of banana/ plantain hybrids. Natural Resources Institute report. Project No. C0361.

Deffenbaugh, L. B. and Walker, C. E. (1989a) Use of the rapid visco-analyser to measure starch pasting properties. Part I: Effect of sugars. *Starch/Stärke* **41**, 461-467.

Deffenbaugh, L. B. and Walker, C. E. (1989b) Comparison of starch pasting properties in the brabender viscoamylograph and the rapid visco-analyser. *Cereal Chemistry* **66**, 493-499.

Dempster, F. D. (1984) Investigations on the pulp and flours of green banana *Musa acuminata* [AAA] *Colla*, with particular reference to breadmaking. PhD Thesis. Department of Food Science, Reading.

Denyer, K. and Smith, A. M. (1992) The purification and characterisation of the two forms of soluble starch synthase from developing pea embryos. *Planta* **186**, 609-617.

Desai, B. B. and Deshpande, P. B. (1975) Chemical transformations in three varieties of banana (*Musa paradisiaca* Linn.) fruits stored at 20°C. *Mysore Journal of Agricultural Sciences* **9**, 634-643.

Dominguez-Puigjaner, E., Vendrell, M., Ludevid, M. D. (1992) Differential protein accumulation in banana fruit during ripening. *Plant Physiology* **98**, 157-162.

Dry, I., Smith, A. M., Edwards, A., Bhattacharyya, M., Dunn, P. and Martin, C. (1992) Characterisation of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. *The Plant Journal* **2**, 193-202.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.

Eggleston, G., Swennen, R. and Akoni, S. (1992) Physicochemical studies on starches isolated from plantain cultivars, plantain hybrids, and cooking bananas. *Starch/Stärke* **44**, 121-128.

Fishwick, M. J. and Wright, A. J. (1980) Isolation and characterisation of amyloplast envelope membranes from *Solanum tuberosum*. *Phytochemistry* **19**, 55-59.

Forsyth, W. G. C. (1980) Banana and plantain. In: *Tropical and subtropical fruits*. Eds. Nagy, S. and Shaw, P. E. 258-278. AVI Publishing Inc., Westport, Connecticut.

French, D. (1984) Organisation of starch granules. In: *Starch, chemistry and technology*. 2nd edition. Eds. Whistler, R. L., BeMiller, J. N. and Paschall, E. F. 184-247. Academic Press Inc., London, U.K.

Fuwa, H., Okuno, K., Asashiba, R., Kikuzaki, H., Asaoka, M., Inouchi, N. and Sugimoto, Y. (1992) Characterisation of high-amylose type endosperm starches of rice plants cultivated in Asia. *Starch/Stärke* **44**, 203-205.

Garcia, E. and Lajolo, F. M. (1988) Starch transformation during banana ripening: the amylase and glucosidase behaviour. *Journal of Food Science* **53**, 1181-1186.

Ghiasi, K., Varriano-Marston, E. and Hoseney, R. C. (1982) Gelatinisation of wheat starch. IV Amylograph viscosity. *Cereal Chemistry* **59**, 262-265.

Gidley, M. J. (1985) Quantification of the structural features of starch polysaccharides by n.m.r spectroscopy. *Carbohydrate Research* **139**, 85-93.

Goering, K. J. (1978) Some anomalies in starch chemistry. Are they due to granule structure? *Starch/Stärke* **30**, 181-183.

Goering, K. J. and Schuh, M. (1967) New starches. III. The properties of the starch from *Phalaris canariensis*. *Cereal Chemistry* **44**, 532-538.

Greenwood, C. T. (1979) Observations on the structure of the starch granule. In: *Polysaccharides in food*. Eds. Blanshard, J. M. V. and Mitchell, J. R. 129-138. Butterworths: London and Boston.

Halmer, P. and Bewley, J. D. (1982) Control by external and internal factors over the mobilization of reserve carbohydrates in higher plants. *Encyclopaedia of Plant Physiology New Series* **13A**, 748-793.

Hari, P. K., Garg, S. and Garg, S. K. (1989) Gelatinisation of starch and modified starch. *Starch/Stärke* **41**, 88-91.

Headly, V. E. and Pfost, H. B. (1968) A comminution equation relating energy to surface area by the log probability method. *Transactions of the ASAE* **11**, 331-338.

Hizukuri, S. (1985) Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. *Carbohydrate Research* **141**, 289-306.

Hizukuri, S. (1986) Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research* **147**, 342-347.

Hizukuri, S., Fujii, M. and Nikuni, Z. (1961) Effect of temperature during germination on the crystalline type of starch in soybean seedlings. *Nature* **192**, 239-240.

Hizukuri, S., Kaneko, T. and Takeda, Y. (1983) Measurement of the chain length of amylopectin and its relevance to the origin of crystalline polymorphism of starch granules. *Biochimica et Biophysica Acta* **760**, 188-191.

Hizukuri, S., Takeda, Y. and Maruta, N. (1989) Molecular structures of rice starch. *Carbohydrate Research* **189**, 227-235.

Hodge, J. E. and Hofreiter, B. T. (1962a) Determination of reducing sugars and carbohydrates. In: *Methods in carbohydrate chemistry*. Vol. I. Eds. Whistler, R. L. and Wolfron, M. L. No. 115. 388-389. Academic Press Inc., New York, U.S.A.

Hodge, J. E. and Hofreiter, B. T. (1962b) Determination of reducing sugars and carbohydrates. In: *Methods in carbohydrate chemistry*. Vol. I. Eds. Whistler, R. L. and Wolfron, M. L. No. 115. 389-390. Academic Press Inc., New York, U.S.A.

Hulme, A. C., Jones, J. D. and Wooltorton, L. S. C. (1964a) Mitochondrial preparations from the fruit of the apple - I. Preparation and general activity. *Phytochemistry* **3**, 173-188.

Hulme, A. C., Jones, J. D. and Wooltorton, L. S. C. (1964b) Mitochondrial preparations from flowers. *Nature* **201**, 795-797.

Imberty, A., Chanzy, H., Pérez, S., Buléon, A. and Tran, V. (1988) The double-helical nature of the crystalline part of A-starch. *Journal of Molecular Biology* **201**, 365-378.

Imberty, A. and Pérez, S. (1988) A revisit to the three-dimensional structure of B-type starch. *Biopolymers* **27**, 1205-1221.

Inouchi, N., Glover, D. V., Takaya, T. and Fuwa, H. (1983) Development changes in fine structure of starches of several endosperm mutants of maize. *Starch/Stärke* **35**, 371-376.

Inouchi, N., Glover, D. V. and Fuwa, H. (1987) Chain length distribution of amylopectins of several single mutants and the normal counterpart, and sugary-1 phytoglycogen in maize (*Zea mays* L.) *Starch/Stärke* **39**, 259-266.

Jenkins, P. J., Cameron, R. E. and Donald, A. M. (1993) A universal feature in the structure of starch granules from different botanical sources. *Starch/Stärke* **45**, 417-420.

Jones, J. D., Hulme, A. C. and Wooltorton, L. S. C. (1965) The use of polyvinylpyrrolidone in the isolation of enzymes from apple fruits. *Phytochemistry* **4**, 659-676.

Kainuma, K. (1988) Structure and chemistry of the starch granule. In: *The biochemistry of plants*. Vol. XIV (Carbohydrates). Ed. Preiss, J. 141-180. Academic Press Inc., London, U.K.

Kayisu, K. and Hood, L. F. (1981) Molecular structure of banana starch. *Journal of Food Science* **46**, 1894-1897.

Kayisu, K., Hood, L. F. and Vansoest, P. J. (1981) Characterisation of starch and fibre of banana fruit. *Journal of Food Science* **46**, 1885-1890.

Ketiku, A. O. (1973) Chemical composition of unripe (green) and ripe plantain (*Musa paradisiaca*). *Journal of the Science of Food and Agriculture* **24**, 703-707.

Kobayashi, S., Schwartz, S. J. and Lineback, D. R. (1985) Rapid analysis of starch, amylose and amylopectin by high-performance size-exclusion chromatography. *Journal of Chromatography* **319**, 205-214.

Kruger, L. H. and Murray, R. (1976) Starch texture. In: *Rheology and texture in food quality*. Eds. deMann, J. M., Voisey, P. W., Rasper, V. F. and Stanley, D. W. 427-444. The AVI Publishing Co., Inc., Westport, Connecticut.

Kugimya, M. and Donovan, J. W. (1981) Calorimetric determination of the amylose content of starches based on formation and melting of the amylose-lysolecithin complex. *Journal of Food Science* **46**, 765-770.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leach, H. W., McCowen, L. D. and Schoch, T. J. (1959) Structure of the starch granule. I. Swelling and solubility patterns of various starches. *Cereal Chemistry* **36**, 534-544.

Lehninger, A. L. (1975) *Biochemistry. The molecular basis of cell structure and function*. 2nd edition. Worth Publishers Inc., New York, U.S.A.

Lii, C.-Y., Chang, S.-M. and Young, Y.-L. (1982) Investigation of the physical and chemical properties of banana starches. *Journal of Food Science* **47**, 1493-1497.

MacDonald, F. D. and Preiss, J. (1985) Partial purification and characterisation of granule-bound starch synthases from normal and *waxy* maize. *Plant Physiology* **78**, 849-852.

MacMasters, M. M. (1964) Microscopic techniques for determining starch granule properties. In: *Methods in carbohydrate chemistry*. Vol. IV (Starch). Eds. Whistler, R. L., Smith, R. J., BeMiller, J. N. and Wolfrom, M. L. No. 50. 233-240. Academic Press Inc., London, U.K.

Manners, D. J. (1968) The biological synthesis of starch. In: *Starch and its derivatives*. 4th edition. Ed. Radley, J. A. 66-90. Chapman and Hall Ltd., London, U.K.

Manners, D. J. (1989) Review paper. Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers* **11**, 87-112.

Miller, B. S., Derby, R. I. and Trimbo, H. B. (1973) A pictorial explanation for the increase in viscosity of a heated wheat starch-water suspension. *Cereal Chemistry* **50**, 271-280.

Morrison, W. R. and Karkalas, J. (1990) Starch. In: *Methods in plant biochemistry*. Vol. II. Eds. Dey, P. M. and Harborne, J. B. 323-352. Academic Press Inc., London.

Morrison, W. R. and Laignelet, B. (1983) An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. *Journal of Cereal Science* **1**, 9-20.

Müller-Röber, B., Sonnewald, U. and Willmitzer, L. (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *The EMBO Journal* **11**, 1229-1238.

Nikuni, Z. (1978) Studies on starch granules. *Starch/Stärke* **30**, 105-111.

Ong, M. H., Jumel, K., Tokarczuk, P. F., Blanshard, J. M. V. and Harding, S. E. (1994) Simultaneous determinations of the molecular weight distributions of amyloses and the fine structures of amylopectins of native starches. *Carbohydrate Research* **260**, 99-117.

Ortiz, R., Ferris, R. S. B. and Vuylsteke, D. R. (1995) Banana and plantain breeding. In: *Bananas and plantains*. Ed. Gowen, S. 110-146. Chapman and Hall Ltd., London.

Palmer, J. K. (1971) The banana. In: *The biochemistry of fruits and their products.* Vol. II. Ed. Hulme, A. C. 65-105. Academic Press Inc., London.

Paredes-López, O., Bello-Pérez, L. A. and López, M. G. (1994) Amylopectin structural, gelatinisation and retrogradation studies. *Food Chemistry* **50**, 411-417.

Preiss, J. (1988) Biosynthesis of starch and its regulation. In: *The biochemistry of plants.* Vol. XIV. Ed. Preiss, J. 181-254. Academic Press Inc., New York, U.S.A.

Preiss, J. (1992) Biology and molecular biology of starch synthesis and its regulation. In: *Oxford surveys of plant molecular and cell biology*. Vol. VII. 59-114.

Preiss, J. and Levi, C. (1980) Starch biosynthesis and degradation. In: *The biochemistry of plants: A comprehensive treatise*. Vol. III. Ed. Preiss, J. 371-423. Academic Press Inc., New York, U.S.A.

Price, N. S. (1995) The origin and development of banana and plantain cultivation. In: *Bananas and plantains*. Ed. Gowen, S. 1-13. Chapman and Hall Ltd., London.

Purseglove, J. W. (1972) *Musaceae*. In: *Tropical crops. Monocotyledons II*. Longman Group Ltd., London.

Rahman, A. R. (1963) Economical method for the production of flour from green plantains. *Journal of Agriculture of the University of Puerto Rico* **47**, 1-10.

Rašper, V. (1969) Investigations on starches from major starch crops grown in Ghana I. - Hot paste viscosity and gel-forming power. *Journal of the Science of Food and Agriculture* **20**, 165-171.

Rašper, V. (1971) Investigations on starches from major starch crops grown in Ghana III. - Particle size and particle size distribution. *Journal of the Science of Food and Agriculture* **22**, 572-580.

Ravindranath, B. (1989) Liquid chromatography. I: Principles and methods. In: *Principles and practice of chromatography*. 193-294. Ellis Horwood Ltd., Chichester, U.K.

Rhodes, M. J. C. (1970) The climacteric and ripening of fruits. In: *The biochemistry of fruits and their products*. Vol. I. Ed. Hulme, A. C. 521-533. Academic Press Inc., London and New York.

Rickard, J. E., Asaoka, M. and Blanshard, J. M. V. (1991) The physico-chemical properties of cassava starch. *Tropical Science* **31**, 189-207.

Rickard, J. E. and Behn, K. R. (1987) Evaluation of acid and enzyme hydrolytic methods for the determination of cassava starch. *Journal of the Science of Food and Agriculture* **41**, 373-379.

Robin, J. P., Mercier, C., Charbonniere, R. and Guilbot, A. (1974) Lintnerised starches. Gel filtration and enzymatic studies of insoluble residues from prolonged acid treatment of potato starch. *Cereal Chemistry* **51**, 389-406.

Rodríguez-Sosa, E. J., González, M. A., de Caloni, I. B. and Parsi-Ros, O. (1977) The preparation of green banana flour. *Journal of Agriculture of the University of Puerto Rico* **61**, 470-478.

Rodriguez-Sosa, E. J. and Parsi-Ros, O. (1984) Ph affects properties of green banana starch. *Journal of Agriculture of the University of Puerto Rico* **68**, 323-329.

Ross, A. S., Walker, C. E., Booth, R. I., Orth, R. A. and Wrigley, C. W. (1987) The rapid visco-analyser: A new technique for the estimation of sprout damage. *Cereal Foods World* **32**, 827-829.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning - A laboratory manual*. Vol. III. 2nd edition. Cold Spring Harbour Laboratory Press, U.S.A.

Samson, J. A. (1986) Banana and plantain. In: *Tropical fruits*. 2nd edition. 139-189. Longman Scientific and Technical, Longman Group Ltd., London, U.K.

Sarko, A. and Wu, H.-C. H. (1978) The crystal structures of A-, B- and C-polymorphs of amylose and starch. *Starch/Stärke* **30**, 73-78.

Schoch, T. J. and Maywald, E. C. (1956) Microscopic examination of modified starches. *Analytical Chemistry* **28**, 382-387.

Schoch, T. J. and Maywald, E. C. (1968) Preparation and properties of various legume starches. *Cereal Chemistry* **45**, 564-573.

See, Y. P. and Jackowski, G. (1989) Estimating the molecular weights of polypeptides by SDS gel electrophoresis. In: *Protein structure: A practical approach*. Ed. Creighton, T. E. 1-21. IRL Press, Oxford, U.K.

Shannon, J. C. and Garwood, D. L. (1984) Genetics and physiology of starch development. In: *Starch, chemistry and technology*. 2nd edition. Eds. Whistler, R. L., BeMiller, J. N. and Paschall, E. F. 25-86. Academic Press Inc., London, U.K.

Shaw, D. J. (1969) *Electrophoresis*. Academic Press Inc., London, U.K.

Shewmaker, C. K. and Stalker, D. M. (1992) Modifying starch biosynthesis with transgenes in potatoes. *Plant Physiology* **100**, 1083-1086.

Sievert, D. and Holm, J. (1993) Determination of amylose by differential scanning calorimetry. *Starch/ Stärke* **45**, 136-139.

Simmonds, N. W. (1962) *The Evolution of the Bananas*. Longman, London.

Simmonds, N. W. (1966) *Bananas*. 2nd edition. Tropical Agriculture Series. Longmans Green & Co., London, U.K.

Smith, A. M. (1990a) Enzymes of starch synthesis. In: *Methods in plant biochemistry*. Vol. III. Ed. Lea, P. J. 93-102. Academic Press Ltd., London.

Smith, A. M. (1990b) Evidence that the 'waxy' protein of pea (*Pisum sativum* L.) is not the major starch-granule-bound starch synthase. *Planta* **182**, 599-604.

Smith, A. M. and Denyer, K. (1992) Starch synthesis in developing pea embryos. Tansley Review No. 39. *New Phytologist* **122**, 21-33.

Smith, A. M. and Martin, C. (1992) Starch biosynthesis and the potential for its manipulation. In: *Biosynthesis and manipulation of plant products*. Vol. III. Ed. Grierson, D. 1-54. Blackie Academic and Professional, U.K.

Sterling, Cl. (1960) Crystallinity of potato starch. *Starch/Stärke* **6**, 182-185.

Sterling, Cl. (1968) The structure of the starch grain. In: *Starch and its derivatives*. 4th edition. Ed. Radley, J. A. 139-167. Chapman and Hall Ltd., London.

Stevens, D. J. and Elton, G. A. H. (1971) Thermal properties of the starch/water system. Part 1. Measurement of heat of gelatinisation by differential scanning calorimetry. *Starch/Stärke* **23**, 8-11.

Stryer, L. (1988) *Biochemistry*. International student edition. 3rd edition. W. H. Freeman and Company, New York, U.S.A.

Suntharalingam, S. and Ravindran, G. (1993) Physical and biochemical properties of green banana flour. *Plant Foods for Human Nutrition* **43**, 19-27.

Swennen, R. and Vuylsteke, D. (1993) Breeding black Sigatoka resistant plantains with a wild banana. *Tropical Agriculture* (Trinidad) **70**, 74-77.

Swinkels, J. J. M. (1985) Starch, sources, chemistry and physics. In: *Starch conversion technology*. Ed. Van Beynum, G. M. A. and Roels, J. A. Marcel Dekker, Inc., New York, U.S.A.

Takeda, Y., Hizukuri, S. and Juliano, B. O. (1987) Structures of rice amylopectins with low and high affinities for iodine. *Carbohydrate Research* **168**, 79-88.

Thompson, A. K., Been, B. O. and Perkins, C. (1974) Effects of humidity on ripening of plantain bananas. *Experientia* **30**, 35-36.

Tyson, R. H. and apRees, T. (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. *Planta* **175**, 33-38.

Ukhun, M. E. and Ukpebor, I. E. (1991) Production of instant plantain flour, sensory evaluation and physico-chemical changes during storage. *Food Chemistry* **42**, 287-299.

Van der Leij, F. R., Visser, R. G. F., Oosterhaven, K., Van der Kop, D. A. M., Jacobsen, E. and Feenstra, W. J. (1991) Complementation of the amylose-free starch mutant of potato (*Solanum tuberosum.*) by the gene encoding granule-bound starch synthase. *Theoretical and Applied Genetics* **82**, 289-295.

Van Soest, J. J. G., de Wit, D. and Vliegenthart, J. F. G. (1994) Retrogradation of potato starch as studied by fourier transform infrared spectroscopy. *Starch/Stärke* **46**, 453-457.

Visser, R. G. F. and Jacobsen, E. (1993) Towards modifying plants for altered starch content and composition. *Trends in Biotechnology* **11**, 63-68.

Visser, R. G. F., Somhorst, I., Kuipers, G. J., Ruys, N. J., Feenstra, W. J. and Jacobsen, E. (1991) Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. *Molecular and General Genetics* **225**, 289-296.

Von Loesecke, H. W. (1950) *Bananas*. 2nd edition. Interscience Publishers Inc. New York and London.

Vuylsteke, D. R. (1989) *Shoot-tip culture for the propagation, conservation and exchange of Musa germplasm*. Practical manuals for handling crop germplasm *in vitro* 2. International Board for Plant Genetic Resources (IBPGR), Rome, Italy.

Wainwright, H. and Burdon, J. N. (1991) Problems and prospects for improving the post harvest technology of cooking bananas. *Postharvest News and Information* **2**, 249-253.

Walker, J. R. L. and Hulme, A. C. (1965) The inhibition of the phenolase from apple peel by polyvinylpyrrolidone. *Phytochemistry* **4**, 677-685.

Walker, C. E., Ross, A. S., Wrigley, C. W. and McMaster, G. J. (1988) Accelerated starch-paste characterisation with the rapid visco-analyser. *Cereal Foods World* **33**, 491-494.

Yau, W. W., Kirkland, J. J. and Bly, D. D. (1979) *Modern size-exclusion chromatography - practice of gel permeation and gel filtration chromatography.* John Wiley and Sons, U.S.A.

Young, R. E., Salminen, S. and Sornsrivichai, P. (1975) Enzyme regulation associated with the ripening in banana fruit. *Colloques internationaux du Centre National de la Recherche Scientifique (C.N.R.S.)* **238**, 271-280.

Zobel, H. F. (1964) X-ray analysis of starch granules. In: *Methods in carbohydrate chemistry.* Vol. IV (Starch). Eds. Whistler, R. L., Smith, R. J., BeMiller, J. N. and Wolfrom, M. L. No. 29. 109-113. Academic Press Inc., New York, U.S.A.

Zobel, H. F. (1984) Gelatinisation of starch and mechanical properties of starch pastes. In: *Starch, chemistry and technology.* 2nd edition. Eds. Whistler, R. L., BeMiller, J. N. and Paschall, E. F. 285-309. Academic Press Inc., London, U.K.

Zobel, H. F. (1988) Starch crystal transformations and their industrial importance. *Starch/Stärke* **40**, 1-7.

Appendix Ia

***Musa* Types Used in the Experimental Programme: Selection of Suppliers**

To enable the characterisation of starch in *Musa* fruits, it was necessary to examine mature green unripe fruits (before any significant starch degradation had occurred during ripening). The unripe fruits were purchased at the earliest possible date after their arrival in the U.K. The best sources of unripe *Musa* fruits were found to be Spitalfields market in London and the Geest packing plant in Chippenham. Both suppliers gave assurance that the fruits had not been subjected to any ethylene treatment (which would trigger the climacteric and induce fruit ripening).

At the market, it was found that very few traders imported cooking bananas to the U.K. In this country, the demand for cooking bananas is small, and the traders did not appear to be importing them on a regular basis. Consequently, it was only possible to purchase a small quantity for examination. In addition, cooking bananas are much smaller fruits than either dessert bananas or plantains. Therefore, the quantity of cooking banana starch and flour was insufficient for use in all of the experiments described.

The Natural Resources Institute has connections with several overseas banana and plantain research establishments (*e.g.* in Honduras and in Nigeria). Through these links, attempts were made to obtain other *Musa* cultivars for use in the experiments. Unfortunately, the fruits were always approaching ripeness, or were fully ripe, when they arrived in the U.K. This degree of ripeness deemed them unsuitable for use in the experimental programme. It was likely that the fruits had been subjected to high temperatures in transit and ripened prematurely. It was not possible to arrange the transportation of unripe *Musa* fruits in a controlled storage environment.

A method of dried pulp transportation was successfully used to obtain further samples. Pulp of different *Musa* types and cultivars was dried in Honduras (the method of drying used by Dr. B. K. Dadzie is detailed in Appendix Ic), before being sent to the U.K. On arrival, these samples were made into flour and their starch contents determined (see Appendix Ic).

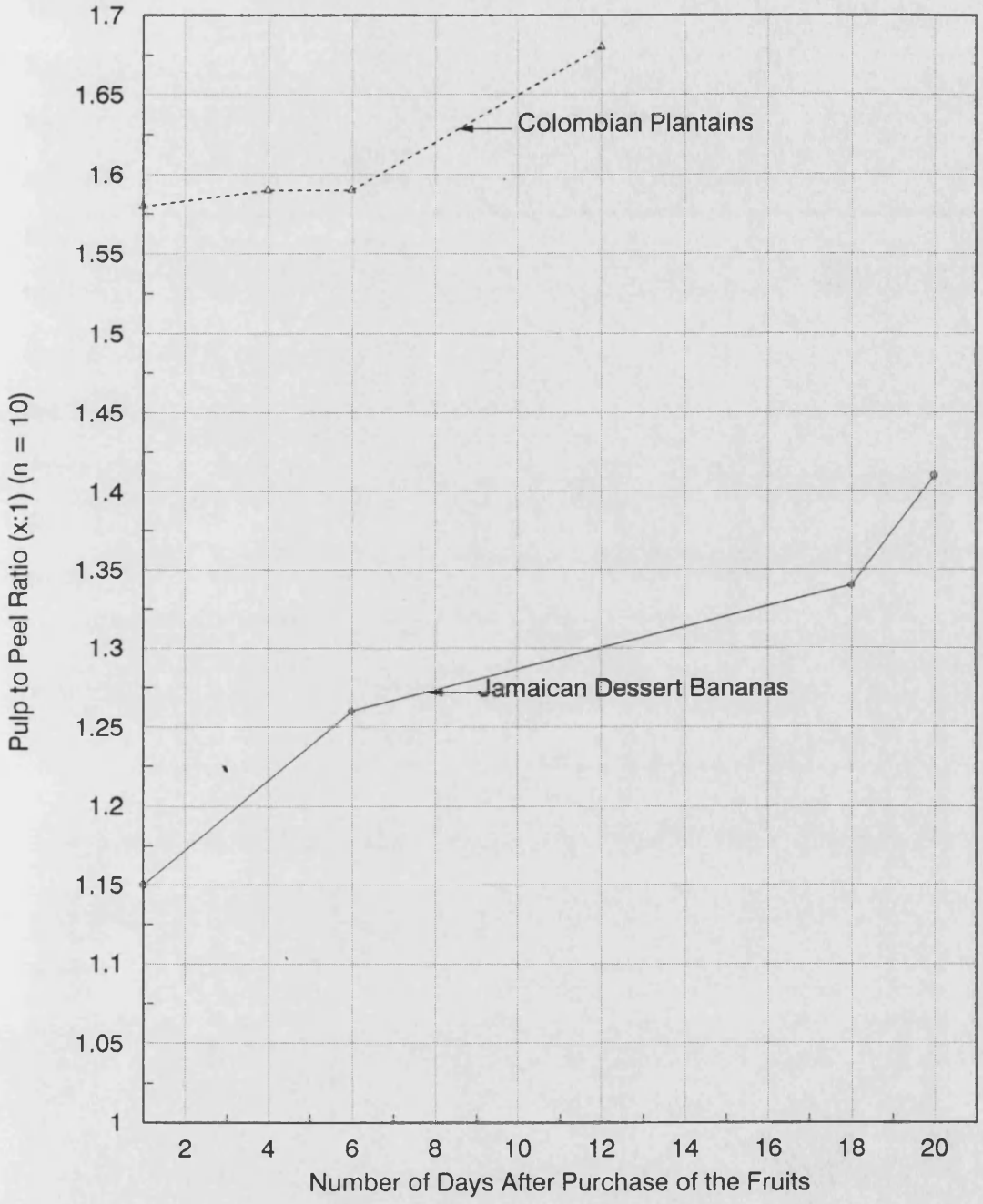
Appendix Ib

Changes in the Pulp to Peel Ratio with Ripening in *Musa* Fruits

The pulp to peel ratio is often used as an index of fruit maturity in bananas and plantains. The pulp to peel ratios of Colombian plantains (genome AAB, Horn) and Jamaican dessert bananas (genome AAA, Cavendish) were determined by hand-peeling the fingers of fruit and weighing the peel and pulp separately.

Plots were made of the pulp to peel ratios of Jamaican dessert bananas and Colombian plantains at different stages of fruit maturity during ripening (Figure I). The pulp to peel ratio increased with increasing fruit ripeness. This was in agreement with Von Loesecke (1950) and Simmonds (1966). In comparison with the values of dessert banana in Figure I, Kayisu *et al.* (1981) reported pulp to peel ratios of green and ripe dessert bananas (genome AAA, Cavendish, 'Valery' var.) of 1.20: 1 and 2.05: 1, respectively.

Figure I Plots of the Changes in the Pulp to Peel Ratio with Ripening in Musa Fruits



Appendix Ic

Starch Contents of Flours of Different Unripe *Musa* Types and Cultivars from Honduras at Different Stages of Fruit Maturity

The FHIA, in Honduras, has had a number of successes with the production of Black Sigatoka resistant tetraploid hybrids, namely FHIA-2 (SH-3486) and SH-3481. The FHIA has been dedicated to the production of improved diploids and primary tetraploids, though the ultimate aim is to develop secondary triploids, via tetraploid x diploid crosses. The FHIA-2 hybrid is being used as a female parent for back crossing to produce secondary triploids (cited by Ortiz *et al.*, 1995). The second back-cross allows the addition of further resistance traits. Besides being seedless, these secondary triploids may retain their leaves longer, their bunches are not prone to weak peduncles and their fruits do not exhibit weak necks which leads to premature bunch and finger detachment when ripe ('finger-drop') (Ortiz *et al.*, 1995). An interesting and valuable finding in *Musa* research has shown that there is no tight linkage between the gene(s) coding for Black Sigatoka resistance and the gene(s) responsible for inferior bunch characteristics (Swennen and Vuylsteke, 1993). So far, no FHIA hybrids have reached a sufficient quality standard to be accepted by the banana industry.

In Table I, the starch contents of flours made from green unripe Honduran *Musa* fruits, with different resistances to the Black Sigatoka leaf spot disease, are shown. The flours were produced by drying cubes of pulp (~1 cm^3) at 76°C in a conventional oven for a few days. The dried cubes of pulp were blended for 30 seconds, then blended again for another 30 seconds after a pause, with a 'Moulinex' coffee grinder. The blended dried pulp was sieved through a 425 μm mesh sieve and stored (according to section 3.2.3) until required. The starch contents of the flours, determined using the glucose oxidase method (section 3.2.5), showed that the flours of the different unripe *Musa* types and cultivars had similar starch contents (Table I).

Table I Starch Contents of Flours of Different Unripe *Musa* Types and Cultivars from Honduras

Cultivar	*Musa* Type and Genome	Black Sigatoka Resistance	% Starch Content (d.w.b.) ($n = 6$)
Grand Naine	Dessert Banana, AAA	S	73.3 ± 3.1
Williams	Dessert Banana, AAA	S	74.6 ± 3.9
Cuerno	Plantain, AAB	HS	75.3 ± 2.9
Bluggoe	Cooking Banana, ABB	R	72.7 ± 3.4
FHIA-01	Banana Hybrid*, AAAB	HR	73.3 ± 3.7
FHIA-02	Banana Hybrid, AAAB	HR	69.5 ± 3.7
FHIA-03	Cooking Banana Hybrid**, AABB	R	66.4 ± 2.8
FHIA-06	Cooking Banana Hybrid, AAAB	IR	73.2 ± 3.5
FHIA-21	Plantain Hybrid, AAAB	R	74.8 ± 3.5
FHIA-22	Plantain Hybrid, AAAB	R	75.1 ± 2.8

± represents Standard Deviation

* FHIA-01 is eaten both as a dessert banana fruit when ripe or as a vegetable (cooking banana) when unripe

** FHIA-03 is eaten both as a dessert banana fruit (when ripe) and as a vegetable

Key: R = Resistant; IR = Intermediate Resistance; HR = Highly Resistant; S = Susceptible; HS = Highly Susceptible.

In Table II, the starch contents of flours made from fruits of two different Honduran *Musa* types (with different resistances to the Black Sigatoka leaf spot disease, see Table I) at different stages of fruit maturity, are shown. The fruits were assessed according to their peel colour score which ranged from 1 (unripe), to 9 (very ripe). The flours were produced by drying cubes of pulp (~1 cm^3) either at 76°C in a conventional oven for a few days (controls), or with an acetone treatment. The acetone treatment involved immersing the cubes of *Musa* pulp (~1 cm^3) in 20% acetone for 12 h. The cubes were then immersed

in progressively higher concentrations of acetone, rising by 10% per immersion, each step lasting 12 h, until a final immersion of 100% acetone was made. The dried cubes of pulp were blended for 30 seconds, then blended again for another 30 seconds after a pause, with a 'Moulinex' coffee grinder. The blended dried pulp was sieved through a 425 μm mesh sieve and stored (according to section 3.2.3) until required. The starch contents of the flours, determined using the glucose oxidase method (section 3.2.5), showed that the flours of the different *Musa* types contained different concentrations of starch, and the concentration of starch in the *Musa* flour samples decreased with increasing ripeness of the fruits from which the flours were made (Table II).

Table II Starch Contents of Flours of Cuerno and FHIA-21 at Different Stages of Fruit Maturity

Sample	Peel Colour Score	% Starch Content (d.w.b.)
Cuerno - Acetone	1	73.0 ± 0.5 (n = 3)
Cuerno - Control	1	73.6 ± 0.3 (n = 3)
Cuerno - Acetone	2	70.7 ± 1.3 (n = 3)
Cuerno - Control	2	70.7 ± 2.2 (n = 3)
Cuerno - Acetone	3	71.1 ± 0.8 (n = 3)
Cuerno - Control	3	67.2 ± 0.2 (n = 3)
Cuerno - Acetone	4	70.8 ± 1.3 (n = 3)
Cuerno - Control	4	68.5 ± 1.4 (n = 3)
FHIA-21 - Acetone	1	69.9 ± 3.4 (n = 3)
FHIA-21 - Control	1	70.2 ± 1.0 (n = 3)
FHIA-21 - Acetone	2	73.4 ± 1.0 (n = 3)
FHIA-21 - Control	2	70.1 ± 2.4 (n = 3)
FHIA-21 - Acetone	9	5.2
FHIA-21 - Control	9	5.6 ± 0.4 (n = 3)

± represents Standard Deviation

Appendix Id

Equation for the Determination of the Starch Contents of the *Musa* Flours (see section 3.2.5 for method)

Calculation of starch in original sample:-

Acid hydrolysis

$$s = \frac{0.9 \times c_3 \times 5000}{d_3} \quad \text{g/g alcohol insoluble material}$$

$$\text{Starch in original sample} = \frac{a \times s \times 1000 \text{ mg/g}}{d_1}$$

where,

s	=	Weight of starch in the alcohol soluble solids.
c_3	=	Weight of sugars in the reaction mixture (µg/0.1 ml).
d_3	=	Weight of sample of alcohol insoluble solids used (200 mg).
0.9	=	Conversion factor from glucose to starch. As the starch values were obtained by reading off from a glucose calibration curve, the values obtained were those of glucose. To convert these values to starch they were multiplied by 0.9, which is the conversion factor from glucose to starch. This is because as the starch is hydrolysed, water is added to each sub-unit, and the ratio of weights of glucose: water is 9: 1. Therefore, the starch value will be nine tenths (0.9) of the corresponding value for glucose.
5000	=	Conversion factor since the 200 mg of alcohol insoluble material from the original sample are dissolved in 500 ml, 0.1 ml is taken, and the absorbance due to reducing sugars in the reaction mixture measured.
a	=	Weight of alcohol insoluble solids obtained after extraction.

d_1 = Dry weight of original sample.

1000 = Conversion factor to convert final answer from g/g to mg/g if c is entered as c x 10^{-6} g. This conversion factor is not required if c is entered as c μg.

The starch contents were expressed on a dry weight basis. Thus the moisture contents of the flours both before and after ethanol extraction were used in the above calculations accordingly.

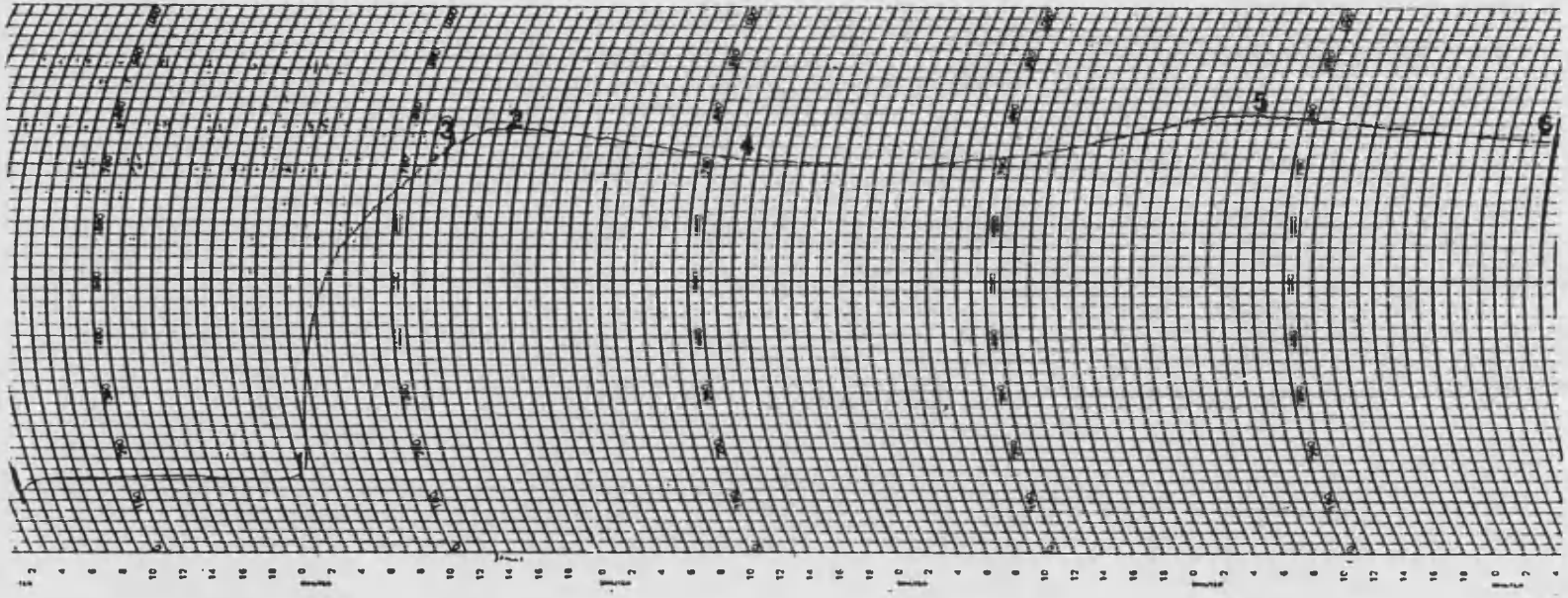
Appendix II

Brabender Viscoamylograms of *Musa* Flours and Reference Starch Samples (see Table 6.3.1).

Brabender viscoamylogram of unripe Dominican Republic dessert banana flour, made on day 2 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 250 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 63.9 ± 2.5 (n = 2); Moisture Content (%, d.w.b) = 8.0 (n = 3)

± represents Standard Deviation

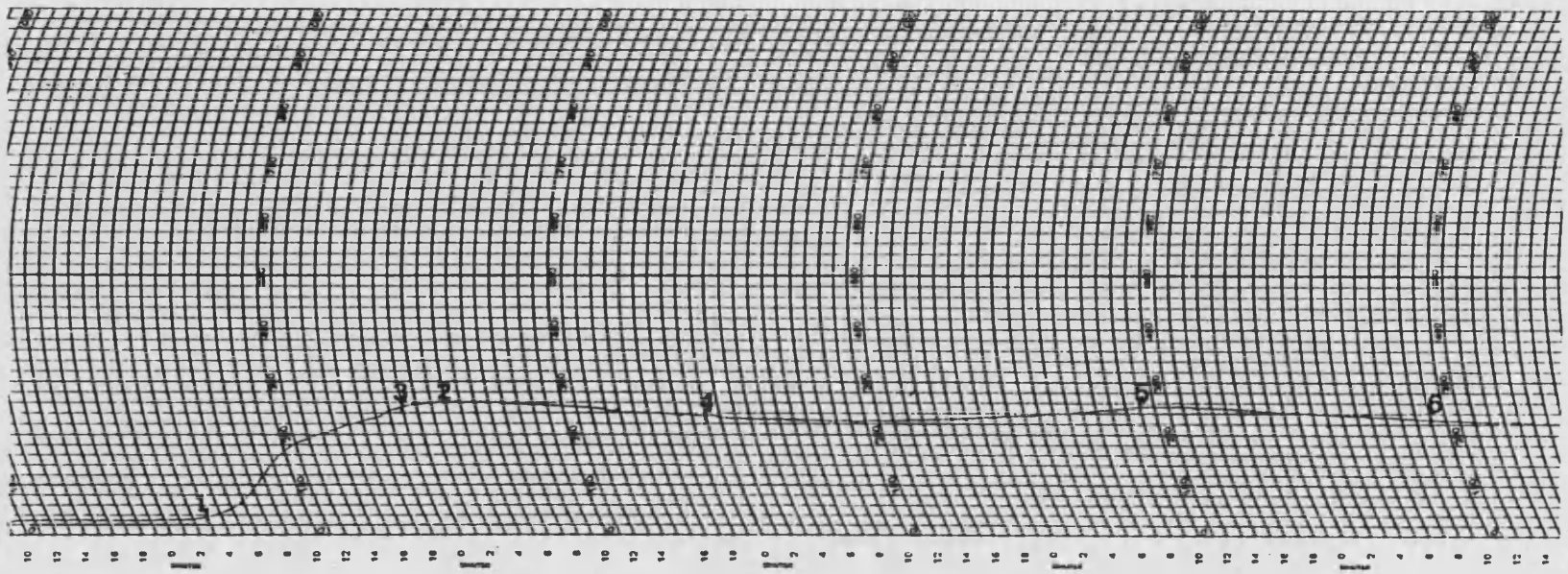


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Jamaican dessert banana flour, made on day 1 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 63.7 ± 1.3 (n = 2); Moisture Content (%, d.w.b) = 3.0 (n = 3)

± represents Standard Deviation

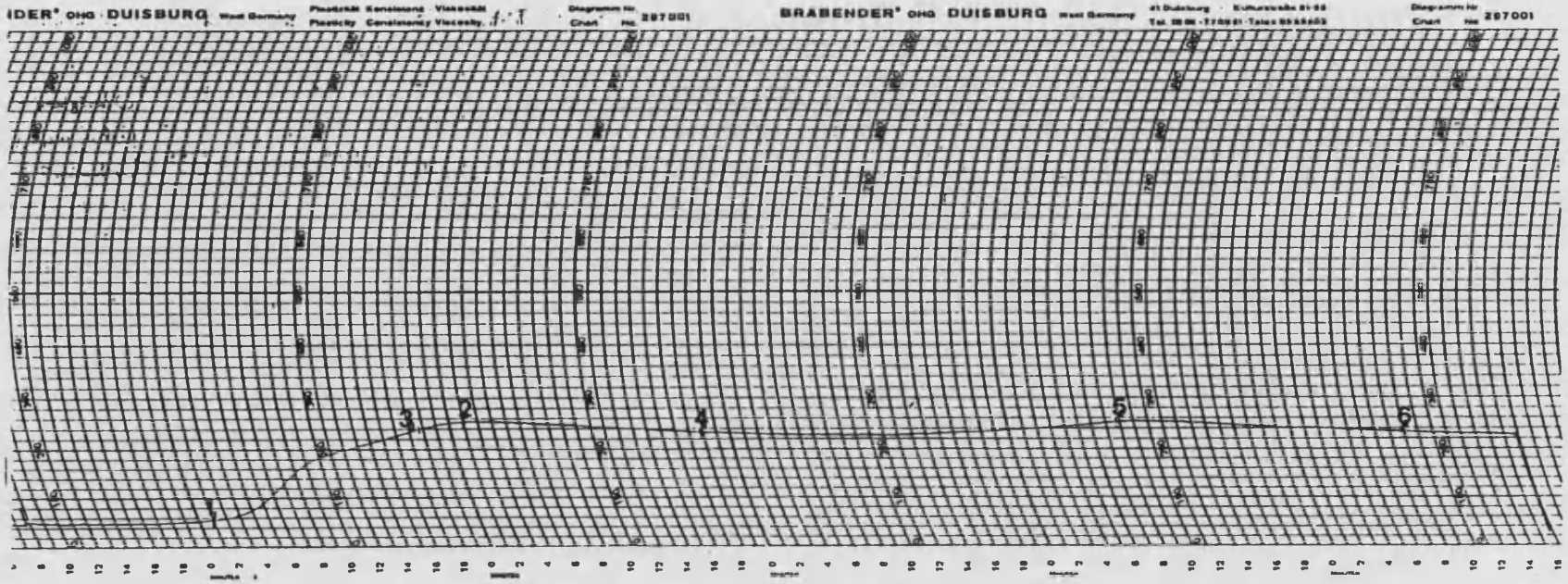


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Jamaican dessert banana flour, made on day 1 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 63.7 ± 1.3 (n = 2); Moisture Content (%, d.w.b) = 3.0 (n = 3)

± represents Standard Deviation

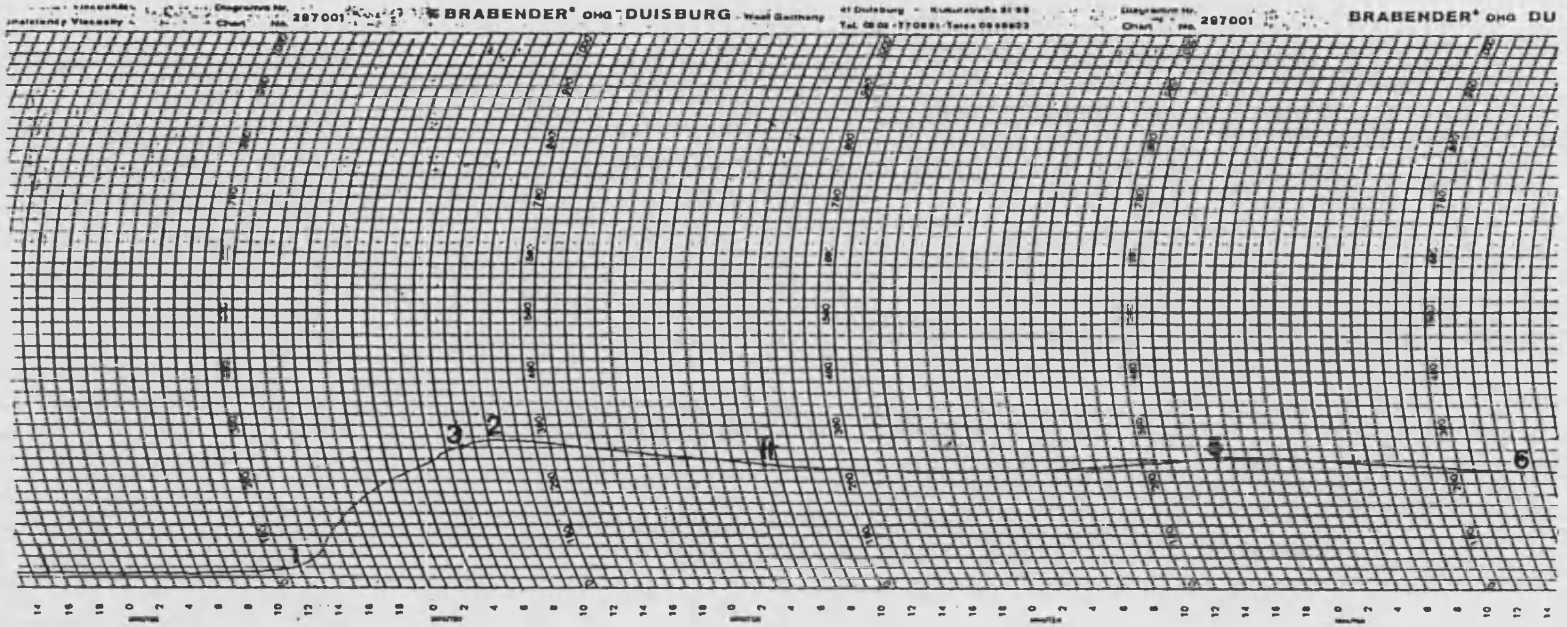


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of ripe Jamaican dessert banana flour, made on day 18 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 59.3 ± 1.2 (n = 2); Moisture Content (%, d.w.b) = 3.7 (n = 3)

± represents Standard Deviation

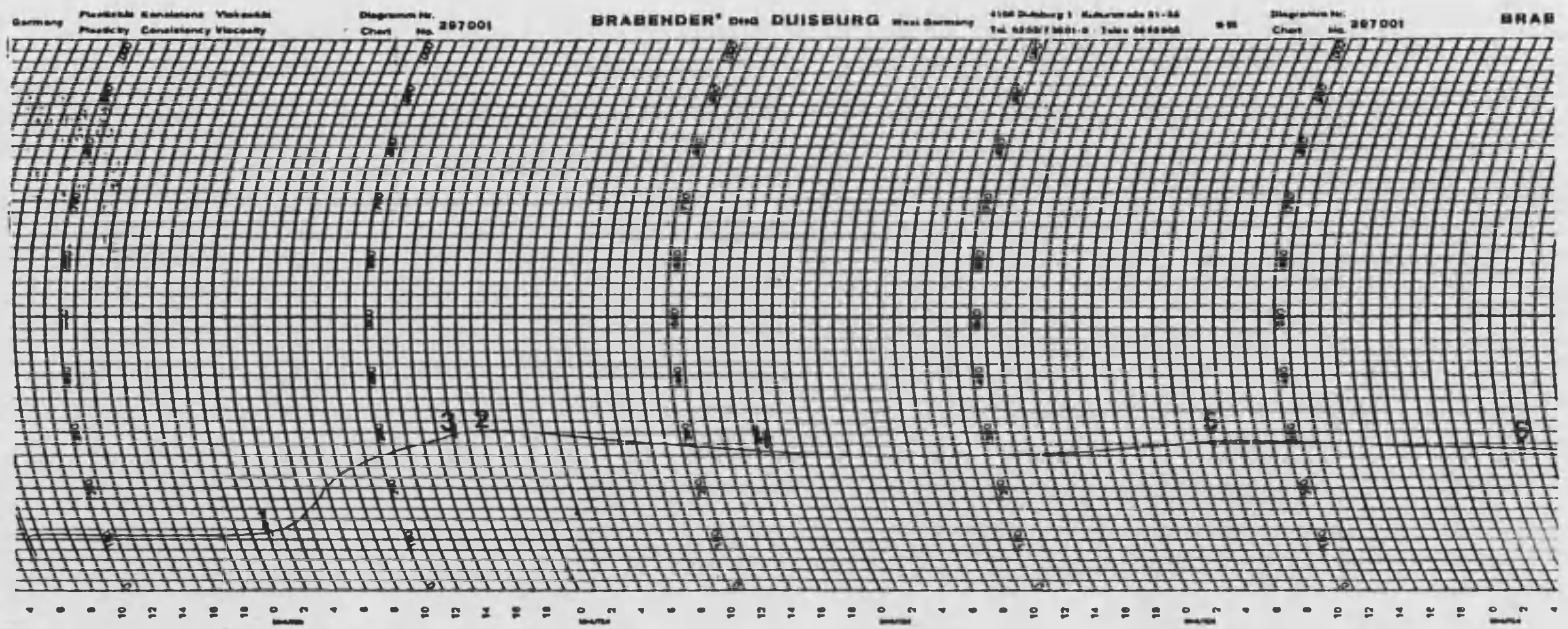


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); 4, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of ripe Jamaican dessert banana flour, made on day 18 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 59.3 ± 1.2 (n = 2); Moisture Content (%, d.w.b) = 3.7 (n = 3)

± represents Standard Deviation

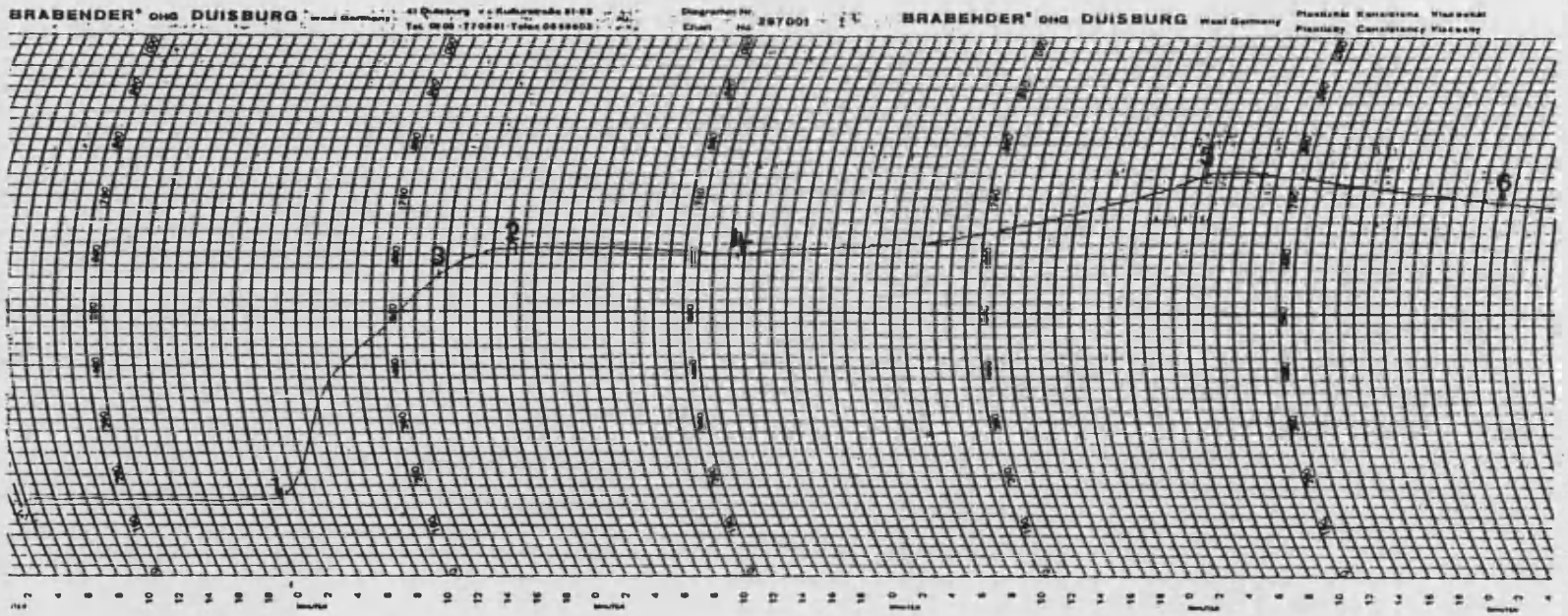


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Dominican Republic plantain flour, made on day 2 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 250 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 68.2 ± 1.0 (n = 2); Moisture Content (%, d.w.b) = 1.5 (n = 3)

± represents Standard Deviation

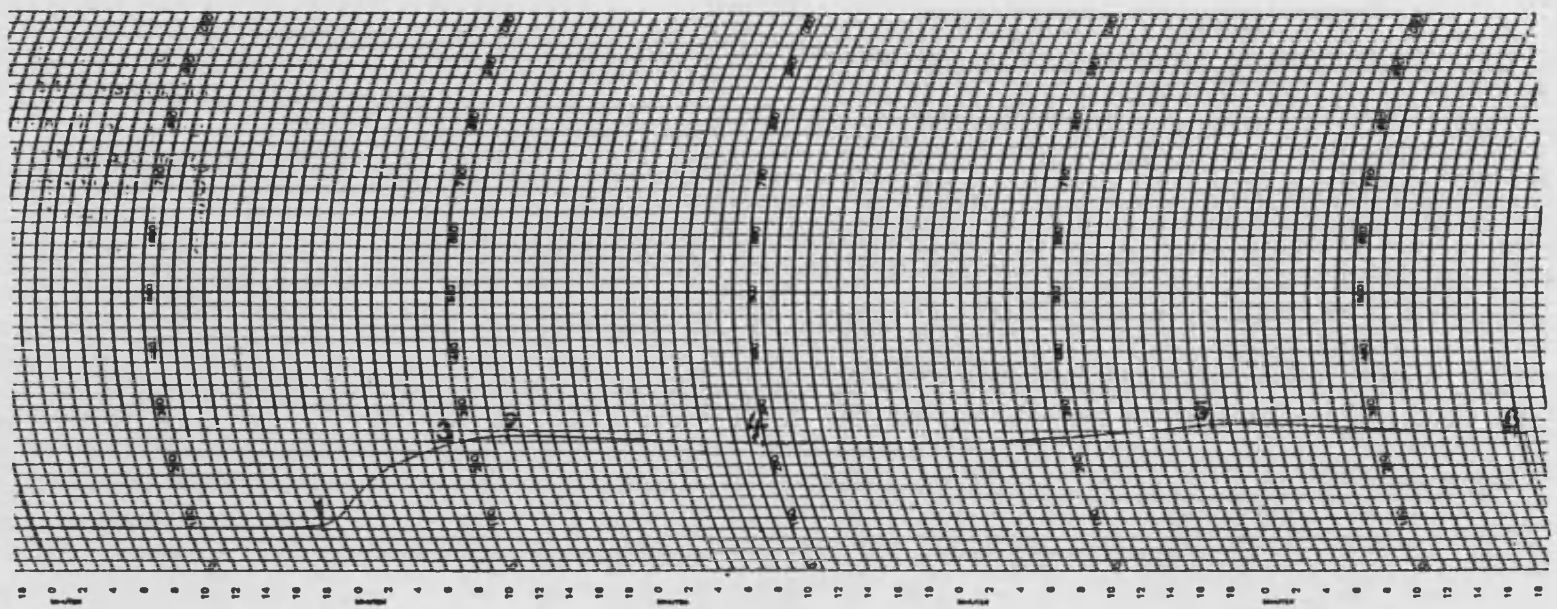


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Colombian plantain flour, made on day 1 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 66.0 ± 2.4 (n = 2); Moisture Content (%, d.w.b) = 2.0 (n = 3)

± represents Standard Deviation

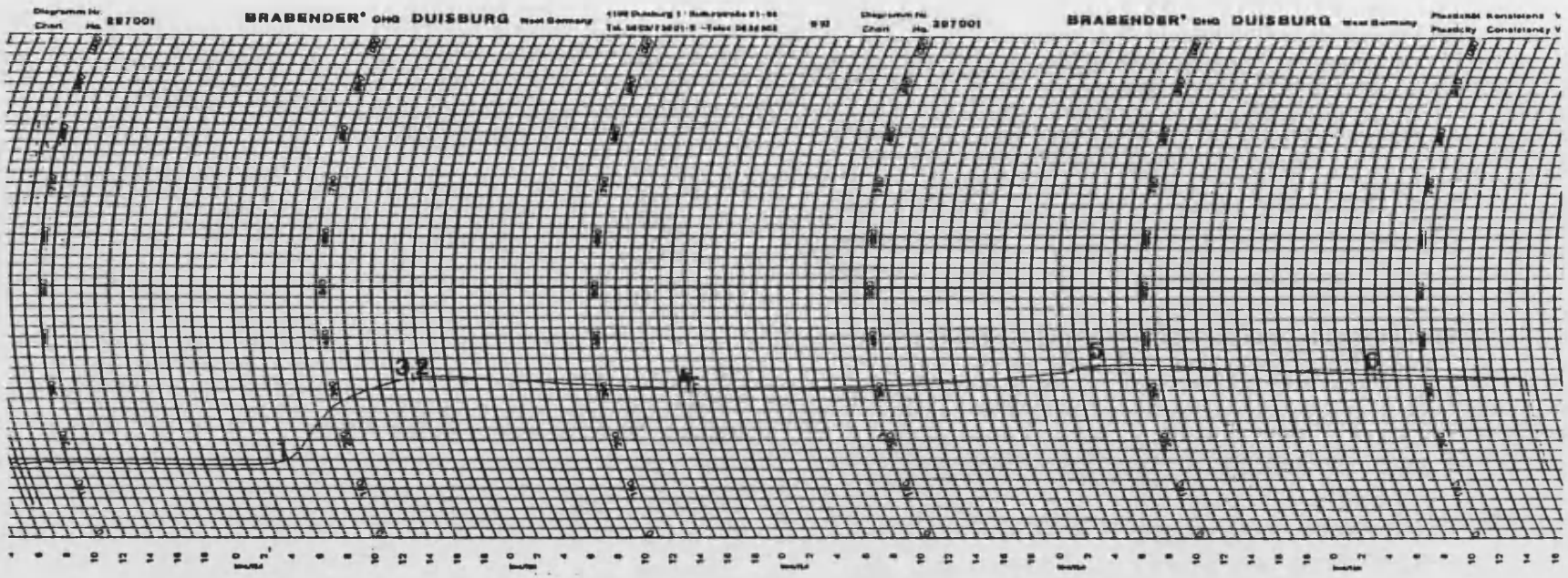


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Colombian plantain flour, made on day 1 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 66.0 ± 2.4 (n = 2); Moisture Content (%, d.w.b) = 2.0 (n = 3)

± represents Standard Deviation

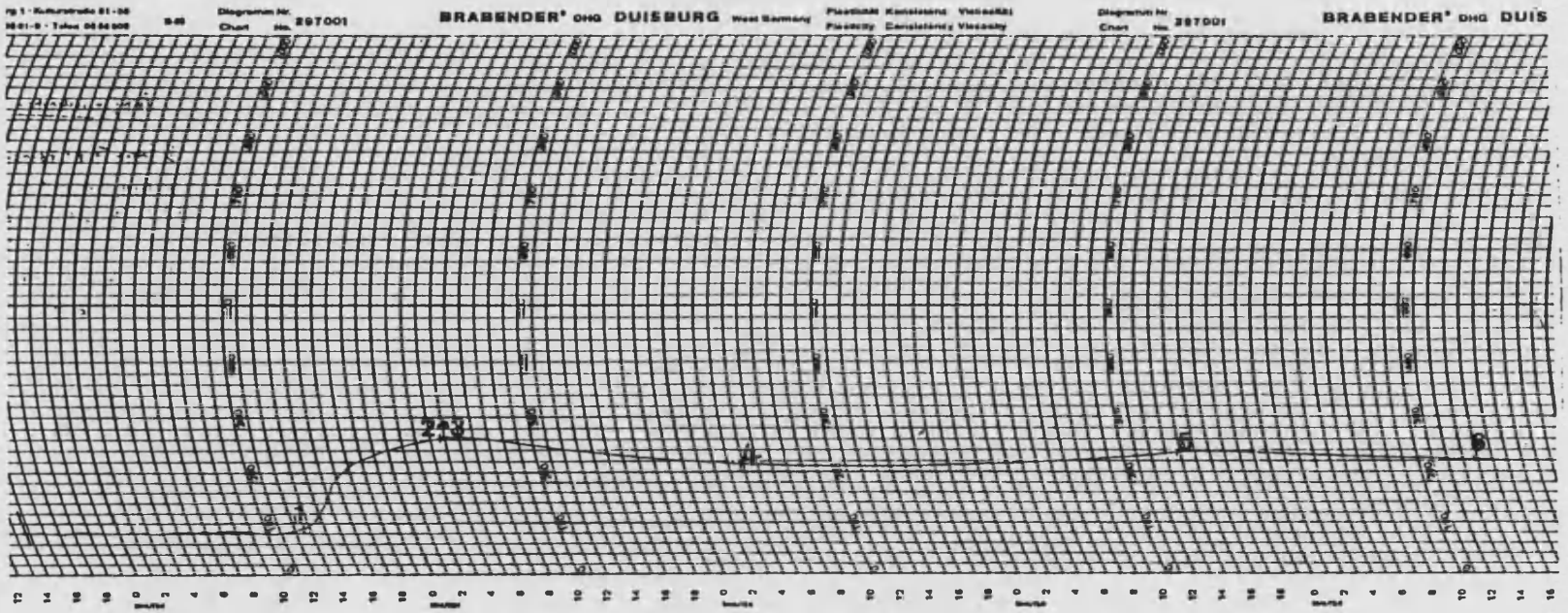


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of ripe Colombian plantain flour, made on day 18 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 61.4 ± 1.1 (n = 2); Moisture Content (%, d.w.b) = 3.3 (n = 3)

± represents Standard Deviation

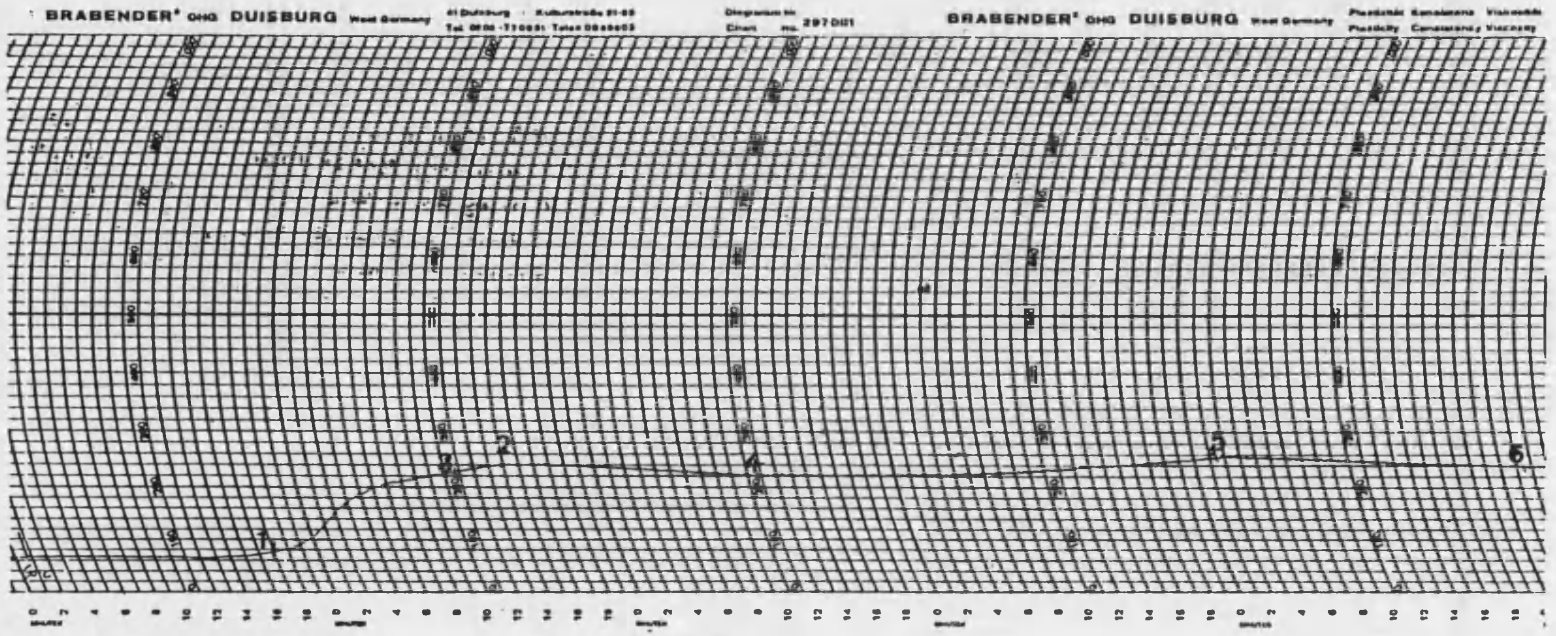


Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Brabender viscoamylogram of unripe Dominican Republic cooking banana flour, made on day 2 after purchase of the fruits. A 5% suspension was used, and the Brabender Viscoamylograph was equipped with a 700 cmg sensitivity cartridge.

Starch Content (%, d.w.b) = 58.8 ± 0.4 (n = 2); Moisture Content (%, d.w.b) = 8.3 (n = 3)

± represents Standard Deviation



Key: **1**, Pasting temperature (T^1); **2**, Peak viscosity (PV); **3**, Viscosity at 95°C (HV^1); **4**, Viscosity after 20 min at 95°C (HV^2); **5**, Viscosity at 50°C (CV^1); **6**, Viscosity after 20 min at 50°C (CV^2).

Appendix IIIa

Recipes for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All chemicals used were supplied by Sigma Chem. Co., U.K., unless otherwise stated.

SDS-PAGE Stock Sample Buffer:

In 10 ml:- 4.8 ml MilliQ H_2O

1.2 ml 0.5 M Tris-HCl, pH 6.8

2 ml 10% SDS

1 ml Glycerol

0.5 ml 0.5% Bromophenol Blue

Running Buffer (Laemmli, 1970)*:*

0.025 M Tris (pH 8.3)

0.192 M Glycine

0.1% SDS

Gel Porosity: 10% Separating Gel (Main Gel) (Sambrook *et al.*, 1989)

In 10 ml:- 4.0 ml Dist. H_2O

3.3 ml 30% Acrylamide Mix

2.5 ml 1.5 M Tris (pH 8.8)

0.1 ml 10% SDS

0.1 ml 10% Ammonium Persulfate

0.004 ml Temed

Gel Porosity: 7.5% Separating Gel (Main Gel) (Sambrook *et al.*, 1989)

In 10 ml:- 4.85 ml Dist. H_2O

2.5 ml 30% Acrylamide Mix

2.5 ml 1.5 M Tris (pH 8.8)

0.1 ml 10% SDS

0.05 ml 10% Ammonium Persulfate

0.002 ml Temed

5% Stacking Gel (Sambrook *et al.*, 1989)

In 5 ml:- 3.4 ml Dist. H_2O

0.83 ml 30% Acrylamide Mix

0.63 ml 1.0 M Tris (pH 6.8)

0.05 ml 10% SDS

0.05 ml 10% Ammonium Persulfate

0.005 ml Temed

SDS-PAGE Coomassie Stain:

In 500 ml:- 325 ml Dist. H_2O

125 ml Methanol (Merck-BDH Co., U.K.)

50 ml Acetic Acid (Merck-BDH Co., U.K.)

0.5 ml Coomassie R250

SDS-PAGE De-stain:

In 2.5 litres:- 1.5 litres Dist. H_2O

750 ml Methanol (Merck-BDH Co., U.K.)

250 ml Acetic Acid (Merck-BDH Co., U.K.)

Appendix IIIb

Recipes for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis prior to Western Blotting

Main Gel Stock Buffer:

	100 ml	Concentration
Tris-HCl, pH 8.8	6.055	0.500 M
20% SDS	0.666	0.133%

Stacker Gel Stock Buffer:

	100 ml	Concentration
Tris-HCl, pH 6.8	1.730	0.143 M
20% SDS	0.575	0.115%
30% Acrylamide:		
Acryl	29.2 g	
Bis	0.8 g	
10% Ammonium Persulfate:	100 mg + 900 µl Dist. H_2O	

Gel Porosity: 7.5% Separating Gel (Main Gel) -

	2 gels (8 ml)
30% Acrylamide	2 ml
Main Gel Buffer	5.956 ml
10% Ammonium Persulfate	0.040 ml
Temed	0.004 ml

3.72% Stacking Gel:

	2 gels (2.5 ml)
30 % Acrylamide	0.31 ml
Stacker Gel Buffer	2.18 ml
10% Ammonium Persulfate	0.0125 ml
Temed	0.0025 ml

Western Blotting Solutions

Western Blotting Buffer:

Tris (Trizma) Base	15 g
Glycine	72 g
Methanol	0.5 litres
Dist. H_2O	4.5 litres

Phosphate Buffered Saline (PBS): (x 10)

	2 litres, pH 7.2
NaCl	170 g
Na_2HPO_4	21.2 g
NaH_2PO_4	7.8 g